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THE OXIDATION-REDUCTION POTENTIAL OF COOKED MEAT MEDIA.

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In a recent paper (1929) on the reducing mechanisms in the meat-media used for the growth of anaërobes we estimated that in meat-tubes freshly prepared as described, although open to the air, the Eh, 1 or 2 cm. from the surface, was of the order of — 0·2 volt. Our reasons for this conclusion were that below this level indigo-carmine appeared to be completely decolorized and parahæmatin reduced to hæmochromogen.

In the coloured meat-hash it is not possible to be sure that the whole of the indigo-carmine is reduced to the leuco-dye, so we have since determined the Eh directly. For this purpose test-tubes 15 × 2·5 cm. were half-filled with neutralized minced meat and enough M/15 phosphate of pH 7·5 added to cover it. Electrodes with a metallic surface of 3 to 4 sq. cm. were embedded in the meat, and the upper ends of the glass tubes supporting them secured at the orifices of the test tubes with cotton-wool packing. Both gilded platinum plates sealed into glass-tubes and iridium mirror electrodes deposited on the outside of glass tubes by the method of Westhaver (1905) were used.

The test-tubes were autoclaved with the electrodes in position, and allowed to cool down in the constant temperature chamber (36° C.) overnight. Next morning they were connected with a 3·5 N KCl calomel half cell by saturated KCl agar bridges and saturated KCl solution. The KCl agar bridges were introduced with aseptic precautions. Observations of the E.M.F. were made at intervals up to 24 hours.

In a weak solution of partially reduced indigo-carmine in an atmosphere of N_2, the iridium and gold electrodes gave readings of E.M.F. which agreed within a millivolt. When, however, one of each was inserted into the same meat-tube and time allowed for the potential to stabilize, sometimes the E.M.F.

agreed, sometimes the iridium electrode afforded a higher reading than the gold electrode, sometimes lower. The discrepancy amounted sometimes to as much as 30 millivolts. A few examples are given below.

Meat-tube.	E.M.F. gold electrode.	E.M.F. iridium electrode.
1	— ·4261	— ·4260
2	— ·4196	— ·4198
3	— ·4405	— ·4653
4	— ·3937	— ·3847
5	— ·4089	— ·4422

All our observations were taken under conditions similar to those in which the tubes are used for anaërobic culture. The tubes were widely open to the air and were not stirred. A meat-tube is a heterogeneous system, and equilibrium in the liquid surrounding the electrodes is but slowly attained by action at the solid surfaces of the pieces of meat. The discrepancies observed are therefore not surprising, and as similar differences in the E.M.F. were obtained when electrodes of the same metal were employed, it is not necessary to assume that they are due to peculiarities of the electrodes.

The E.M.F. of different tubes in two experiments is set out below. There was some irregularity in the first 1 to 2 hours after the agar bridges were inserted. The tubes had to be removed from the incubator for this purpose, and they took some time to arrive at equilibrium after the disturbance. There was little change in the potential between 2 and 5 hours; subsequently the potential became less negative to the extent of 20 to 50 millivolts after 24 hours. This is due to absorption of O_2 at the wide open surface.

Observations with Iridium Electrodes. Temperature 36° *C.*

Time in mins.	E.M.F. of tubes. I.	II.	III.	IV.	VI.	VIII.	Mean E.M.F.	Mean Eh.
0	—·4398	—·4439	—·4261	—·4141	—·4143	—·3912	—·4215	—·1745
28	—·4338	—·4308	—·4256	—·4158	—·4211	—	—·4254	—·1780
48	—·4248	—·4278	—·4324	—·4130	—·4221	—·3937	—·4190	—·1720

Observations with Gold Electrodes. Temperature 36° *C.*

Time in hours.	E.M.F. of tubes. I.	II.	III.	IV.	Mean E.M.F.	Mean Eh.
0·5	— ·4302	— ·4113	— ·4383	— ·3981	— ·4195	— ·1725
1·0	— ·4261	— ·4196	— ·4405	— ·3927	— ·4195	— ·1725
2·5	— ·4283	— ·4247	— ·4339	— ·4002	— ·4215	— ·1745
4·5	— ·4230	— ·4221	— ·4190	— ·4130	— ·4193	— ·1723
24	— ·4150	— ·4010	— ·4160	— ·3580	— ·3980	— ·1510

Most of this batch of tubes stabilized at an E.M.F. round about — ·420 volts against the 3·5 N KCl calomel half cell, indicating an Eh of about — ·174. In others, when fresh, it was as low as —·2.

When the contents were stirred up the potential at once dropped some 50 millivolts, and it took several hours before the previous reading was again reached.

There is evidently a reversible system present which poises the oxidation-reduction potential in this region. In our previous paper we concluded that this was parahæmatin-hæmochromogen, a reversible system studied by Conant (1923). His earlier observations were not very satisfactory, but from the figures he gives we calculate that when equal concentrations of oxidants and reductants are present the Eo at pH 7·5 would be about — 0·1 volt. In a later paper, Conant, Alles and Tongberg (1928) found the Eo for equal concentration of oxidized and reduced pyridin-hæmochromogen to be — ·20 at pH 9·15, and the value computed for Eo at pH 7·5 becomes — ·101 volt, a figure with which Conant's earlier observations on protein-hæmochromogen are not inconsistent.

If the hæmochromagen complex in cooked meat behaves as pyridin-hæmochromogen 95% reduction should occur when the Eh is — ·179 volt, at pH 7·5. The Eh when 99% is reduced would be — ·22. This is consistent with the appearance of the lower half of our meat-tubes in which the large proportion of the parahæmatin is reduced, for the colour becomes little if at all pinker on adding a powerful reducing agent such as $Na_2S_2O_4$.

Our conclusion from colorimetric observations that the Eh in the depth of meat-tubes approaches — ·2 volts is confirmed by electrometric determinations.

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THE OXIDATION-REDUCTION POTENTIAL OF COOKED-MEAT MEDIA FOLLOWING THE INOCULATION OF BACTERIA.

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IN recent papers (1929, 1930) we have shown that O_2 does not penetrate far into culture-tubes of cooked meat prepared as we described, because it is fixed by the unsaturated fatty acids of the lipins in the meat. In addition to this auto-oxidation, which is catalysed by the hæmatin present, the pieces of meat contain a reducing substance, and the reduction potential is poised by the presence of the reversible system para-hæmatin-hæmochromogen.

If, therefore, such a medium is sown with bacteria, their growth cannot influence the redox potential unless it gives rise to reducing substances of considerable intensity. In that case a drift to a more negative level may be expected, but unless other reversible systems are formed either from the medium or by the disintegration of the bacteria it is doubtful whether reproducible measurements could be obtained.

As will be seen later, but slight effect upon the Eh of meat-tubes was observed unless the microbes produced either nascent hydrogen or sulphides from the meat.

A gradual drift towards the negative potential of the hydrogen electrode was observed in media inoculated with a mixture of soil organisms by Gillespie (1920). He found that *B. subtilis* and *B. mycoides* did not produce such low potentials as soil organisms, but that *B. coli* grown in peptone broth containing 1% glucose afforded an Eh closely approaching that of the hydrogen electrode and sometimes exceeding it.

Cannan, Cohen and Clark (1926) studied the potential-time curves of yeast suspensions and also of milk sown with *B. subtilis*, a streptococcus, *B. fluorescens* and *B. coli* over 12 hours. The Eh fell at very different rates, and in the case of the last two, reached $-0{\cdot}3$. When *B. coli* was grown in peptone-water containing 0·5% glucose and buffered with phosphate, the Eh reached the potential of the hydrogen electrode in 10 hours and thereafter slightly exceeded it. They comment on the occurrence of hydrogen over-voltage, notwithstanding that the presence of H_2 evolved from the decomposition of the sugar was less than half an atmosphere. A similar result was obtained with glucose media sown with *B. coli* by Aubel and Genevois (1927). Cohen (1928) also observed hydrogen over-voltage with semifluid media inoculated with gas-forming anaërobes. Coulter (1928) found that when *B. typhosus* was sown into broth

without added sugar the Eh only fell to that of the sterile broth de-aërated with N_2.

Coulter used pure gold electrodes; the other observers employed bright platinum electrodes.

METHODS.

The preparation of the meat-tubes and the technique employed to arrive at the Eh are described in our previous papers (Lepper and Martin, 1929, 1930). We emphasize here, however, that the tubes are open to the air, and that the medium, containing but a trace of broth, is free from sugar and buffered with M/15 phosphate solution. We employed large electrodes of heavily gilded platinum or thick mirrors of iridium deposited on the outside of glass tubes. Usually one of each was embedded in each tube.

After autoclaving with the electrodes in position, the meat-tubes were allowed to cool down to the temperature of the thermostat. The agar bridges were inserted and the E.M.F. against a 3·5 N KCl calomel cell observed. The tubes were then inoculated with a large seeding of the various microbes and readings taken at intervals of half an hour for 10 hours and upwards.

OBSERVATIONS WITH AËROBES.

Staphylococcus aureus, *Streptococcus fæcalis*, an anaërobic streptococcus, *B. pyocyaneus*, *B. coli*, *B. paratyphosus A*, *B. paratyphosus B* and *B. proteus vulgaris* were used.

With the exception of the two last the propagation of all these organisms had very little effect upon the Eh of this medium. During the first 10 hours the potential remained remarkably steady and the extreme variation did not exceed ± 10 millivolts (see Fig. 1). During the next couple of days in some of them the potential slowly became 50 millivolts more negative.

In the tubes sown with *B. paratyphosus B* and *B. proteus* the potentials shortly began to drift, and in the course of 24 hours reached an Eh of — 0·4 volts. During the next 24 hours in some tubes it slowly rose again to — 0·35 volts, in others it remained stationary.

Both of these organisms disrupt proteins and give rise to sulphides. The contrast in the behaviour of *B. paratyphosus A* and *B. paratyphosus B* is exemplified in the chart (Fig. 1).

OBSERVATIONS WITH ANAËROBES.

B. welchii, *B. enteritidis sporogenes*, *B. chauvoei*, *B. œdematiens* and *B. tetani* were used. Generally three tubes were inoculated with each organism.

With the exception of *B. tetani*, all of these anaërobes form gas abundantly in the meat medium. Rather less than half of the gas is H_2. When we employed iridium electrodes all meat-tubes inoculated with anaërobes showed a rapid negative drift coincident with the appearance of gas-bubbles, and in a few hours the potential of the hydrogen electrode was approached or even exceeded. This

result is the same as that observed by others, who used platinum electrodes, whenever gas was formed in the media.

In the tubes inoculated with *B. welchii* and *B. enteritidis sporogenes* the potential began to fall in ½ hour, and in 3 hours reached or exceeded that of the hydrogen electrode (see Fig. 1). Tubes sown with *B. œdematiens* and *B. chauvoei* behaved similarly but the fall occurred later and was less rapid. Except in the tubes sown with *B. enteritidis sporogenes*, in which the medium was liquefied, the Eh remained about the same level, — 0·45, for 2–5 hours. After 36 hours it had risen to — 0·43 ± 10 millivolts.

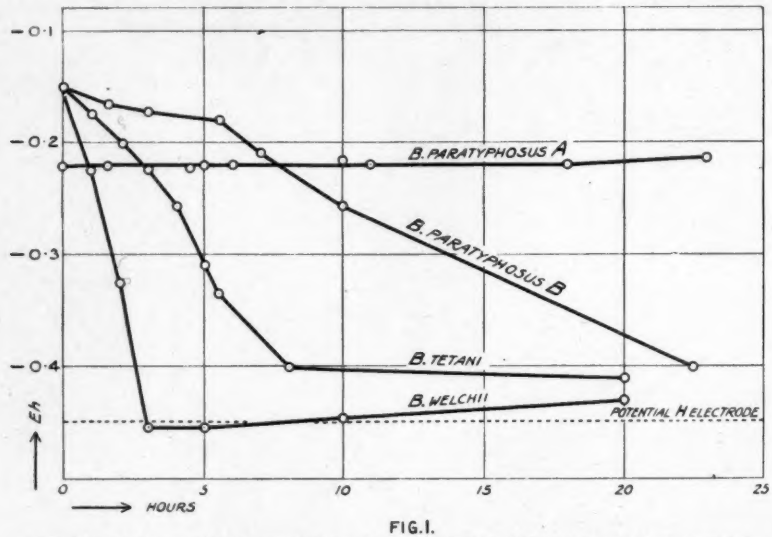

FIG. I.

Time-potential graph of meat medium inoculated with different bacteria at time O.*

In tubes sown with *B. tetani* no sharp fall occurred, but a steady drift to within 50 millivolts of the potential of the hydrogen electrode occurred in the course of 8 hours. Evolution of gas was not obvious, but H_2S was produced.

THE INFLUENCE OF THE METAL OF WHICH THE ELECTRODE IS COMPOSED UPON THE MEASUREMENT OF Eh OBTAINED.

In tubes sown with microbes in which there was not obvious evolution of gas, the E.M.F. at the gold electrode and at the iridium electrode agreed within a few millivolts. In two tubes each containing a gold and an iridium electrode, which were inoculated with *B. tetani* and observed for 60 hours, the discrepancy was never greater than 2 millivolts. During this period the Eh fell steadily from — 0·180 and — 0·163, the Eh of the medium, to — 0·394 and — 0·356 respectively.

* In this and Figs. 2 and 3 the dotted horizontal line represents the potential of the H electrode at the beginning of the experiments.

In tubes sown with gas-forming anaërobes such as *B. welchii* and *B. œdematiens* the readings of E.M.F. afforded by the gold and iridium electrodes were, however, quite different. The rapid fall of about 0·2 volts coincident with the formation of gas-bubbles always observed with iridium electrodes, was absent with gold electrodes. Although nascent hydrogen was produced, the gold electrode failed to register any significant change in E.M.F. (see Fig. 2).

The iridium electrode affords the truer measure of the reduction potential in the medium, for to set free nascent hydrogen requires a potential more negative than the hydrogen electrode. Moreover, the strongly electro-negative dyes, neutral red and safranin, if added to the medium, are decolorized by the action of *B. welchii*.

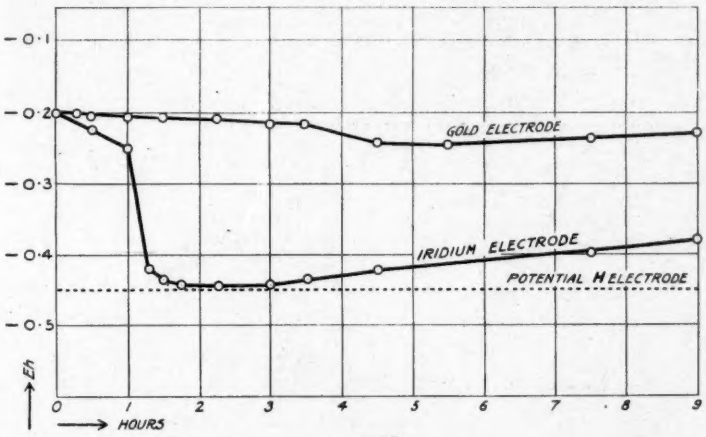

FIG. 2.

Time-potential graph of meat medium inoculated with *B. welchii* at time O. El measured by gold and iridium electrodes.

Acting upon a suggestion of Anderson (1924), we endeavoured to eliminate the production of H_2 by *B. welchii* by adding 1% of KNO_3 to the medium. We were not successful in preventing the formation of gas altogether, but reduced its rate. Gas-formation occurred for a short period only. During this time the Eh measured by the iridium electrode dropped to that of the H_2 electrode, but in the course of 4 hours it rose again and coincided with that afforded by the gold electrode. Meantime the potential at the gold electrode had varied but little. In Fig. 3 is shown the potential time curve of one of these experiments.

The explanation of the different behaviour of electrodes of gold and iridium when gas is evolved would appear to be that, as the formation of hydrogen by the microbes is an irreversible process and gold being relatively insensitive to H_2, there is no reversible oxidation-reduction system present with a more negative range than parahæmatin-hæmochromogen for the gold electrode to come into electro-chemical equilibrium with.

This interpretation is supported by the fact that when the more electronegative dye safranin was added to the medium so as to make a concentration of about ·01%, in one hour after inoculation with *B. welchii* the E.M.F. afforded by the iridium electrodes fell as usual to that of the hydrogen electrode, but the potential of the gold electrodes, instead of remaining stationary, also dropped steeply until it was poised by the safranin around — ·33 volts. The safranin appeared to be entirely absorbed by the meat.

The iridium electrode behaves otherwise, because it becomes charged with H_2 from the solution and functions as a hydrogen electrode. The reason for hydrogen over-voltage noticed by Gillespie (1920) and by Cannan, Cohen and Clark (1926), which also occurred in some of our experiments, is not obvious. The gas evolved contains less than 50% H_2, but the conditions under which it

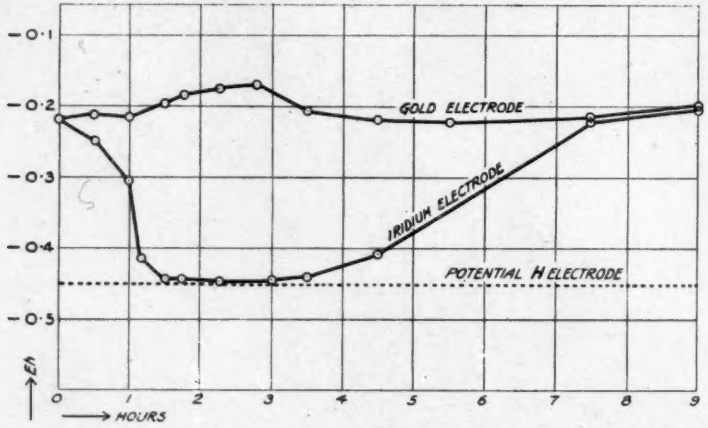

FIG. 3.

Time-potential graph of meat medium + 1% KNO_3 inoculated with *B. welchii* at time O. El measured by gold and iridium electrodes.

is formed are conducive to supersaturation of the solution. Moreover, it is atomic H which is first separated, and this may find access to the electrode direct.

The steady fall in potential when an anaërobe like *B. tetani* is grown in our meat-medium requires another interpretation. There is no obvious gas-formation, and the E.M.F. afforded by the gold electrodes is in this case in agreement with that given by the iridium electrodes. H_2S and organic sulphides are formed and the Eh slowly drops to below — 0·4. We may suppose either that, as Cannan, Cohen and Clark (1926) suggested, soluble reducing substances capable of composing a reversible system are generated, or that some of the organic —S–H compounds formed from the proteins of the meat act upon the electrode like cysteine does. This sulphur-containing amino-acid, although it does not form part of a truly reversible oxidation-reduction system, has been shown by Dixon and Quastel (1923), and later by Michaelis and Flexner (1928),

to afford a definite negative potential varying according to its concentration and the pH of the solution.

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THE EFFECT OF ERGOTAMINE ON CARBOHYDRATE METABOLISM AND ON THE STOMACH.

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THE effect of ergotamine on carbohydrate metabolism has been investigated in two directions:

I. By experiments on normal animals it has been shown to prevent glycogenolysis (or new sugar formation) by paralysing the sympathetic supply to the liver. Thus it abolishes the hyperglycæmia of adrenalin and post-pituitary extract and augments the hypoglycæmic action of insulin (Burn, 1923; Coelto, 1928; Lawrence, 1925).

II. By experiments on normal and diabetic men it has been found by some observers (Loeffler, 1927) to lower the hyperglycæmia after the ingestion of carbohydrate, usually glucose, given by mouth. For this reason it has been said to "increase carbohydrate tolerance," and has been recommended in the treatment of diabetes (Moretti, 1927), on theoretical rather than practical grounds. It therefore seems worth recording some observations which explain its action in this respect.

Ergotamine Checks Glycogenolysis in Diabetics as in Normals.

This action is more easily demonstrated in diabetics with hyperglycæmia than in normal animals with a normal blood-sugar. When the action of insulin is over in severe diabetics the blood-sugar rapidly rises, presumably

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from glycogenolysis in the liver. This hyperglycæmia is checked by ergotamine, as the following experiment shows: A maximal dose of ergotamine was given to a severe diabetic (0·5 mg. = 1 c.c. Femergin Sandoz) at a time when the blood-sugar was rising, and its effect on the blood-sugar concentration was observed every half-hour. The pulse-rate was also noted as a check on the strength and length of activity of the drug.

	Hours after insulin.			Hours after ergot.				
	7.	9.	10.	½.	1.	1½.	2.	2½.
Blood-sugar mg. %	227	284	313	296	287	296	305	316
Pulse-rate	—	—	76	48	50	54	58	56

Ergotamine clearly reduced the hyperglycæmia, though only to a small extent. When the action of the drug began to wear off in about an hour, as the pulse-rate shows, the blood-sugar concentration continued to rise again. It is impossible to give larger doses of ergotamine on account of the unpleasant symptoms described later. It has been noted also in animals that almost lethal doses have to be given to reduce the blood-sugar to any great extent.

Ergotamine Lowers the Blood-Sugar Curve in Normals and Diabetics.

Table I shows the effect of ergotamine on the blood-sugar curve in cases of normal and abnormal carbohydrate metabolism. Other experiments than these were carried out and in every one the result was the same—a lowering of the blood-sugar curve, an apparent "increase in carbohydrate tolerance." The effect of a dose of glucose, usually 50 gm., by mouth was observed in the control experiment, and next day ergotamine (0·5 mg.) was injected subcutaneously 5 minutes before the same dose of glucose. The time of action of the ergotamine was also followed by its effect on the pulse-rate. In half to one hour the pulse-rate was lowered on the average by 20 beats per minute, and had usually recovered partially in 2 hours, and almost completely in 3 or 4 hours.

TABLE I.

		Hours after glucose.					
		0.	½.	1.	1½.	2.	3.
Normal	Control	115	172	156	135	—	—
	Ergotamine	116	110	114	107*	—	—
Diabetic	Control	266	304	370	366	330	—
	Ergotamine	267	247	255*	—	—	—
Hyperthyroid 1	Control	110	183	228	239	219	—
	Ergotamine	103	120	127*	114	122	—
Hyperthyroid 2	Control	116	185	247	243	256	229
	Ergotamine	115	120	122	104	143	174

* Vomited.

It is clear in every case that ergotamine lowered the blood-sugar curve. Most subjects felt sick and three actually vomited, losing in this way more than half the sugar they had eaten. This effect on the stomach is obviously

the most probable mode of action of ergotamine in lowering the blood-sugar curve, and further evidence of this is considered in the next section. In the second case of hyperthyroidism the blood-sugar remained at the fasting level for the first hour and a half, but rose during the second and third hours when the action of ergotamine had worn off. This also suggested that ergotamine might delay the normal *absorption* of sugar, apart from actual vomiting.

Ergotamine Delays the Passage of Sugar through the Stomach.

The literature contained no account of the action of ergotamine on the stomach. It might have been imagined that, being a sympathetic depressor, it would hasten the emptying of the stomach by an unopposed vagus action. However, it was clearly shown by x-ray investigation of three normal individuals that ergotamine greatly delayed the passage of a barium meal, particularly in the early stages of its action.

It seems clear, therefore, that the supposed action of ergotamine in "increasing carbohydrate tolerance" is really due to the delay of the sugar meal in the stomach. An attempt was made to administer the glucose by duodenal tube with and without ergotamine, but no clear results were obtained, and the possibility remains that ergotamine may affect the blood-sugar curve apart from its action on the stomach.

I had originally hoped ergotamine might help to differentiate various forms of abnormal blood-sugar curves, due perhaps to liver defects or an overactive sympathetic system. The action of ergotamine on absorption, however, makes such a test impossible.

Other Observations with Ergotamine.

General effects.—During these experiments the following effects were also noted. The pulse was in every case slowed—in one case from 80 to 48 beats per minute. It became thin and smaller in volume, and the blood-pressure, both systolic and diastolic, was lowered by about 10 mm. of mercury. Dizziness and headache were produced in most experiments and a blue, cyanotic appearance of the face. Nausea of varying degree was present in nearly every case and actual vomiting in about half.

Therapeutic trial in diabetes and Graves's disease.—The dose given was the one usually recommended in obstetrical work, *i. e.* 0·5 mg. It was given subcutaneously in most cases, but sometimes by mouth.

In diabetes, as might be expected, it had a slight but definite action in reducing glycosuria and the blood-sugar, but its action was very short-lived. When given along with insulin the hypoglycæmic action of the latter was increased, though not enough to be of any practical value. It could not in any way replace insulin.

In Graves's disease it seemed reasonable to try whether its action as a sympathetic depressor might be of any clinical value. Dr. East and myself therefore tried it, but without any beneficial result except a slight and transient

slowing of the pulse; and the unpleasant effects above described led to the conclusion that its use was more harmful than beneficial in these two diseases.

SUMMARY.

1. Ergotamine diminishes the hyperglycæmia from endogenous sources in diabetics.

2. Ergotamine diminishes alimentary hyperglycæmia in normals and diabetics. This action is mainly due to delay of the ingested carbohydrate in the stomach.

3. Various unpleasant phenomena due to the action of ergotamine are noted. Clinical trial leads to the conclusion that the drug can be of no practical value in the treatment of diabetes or Graves's disease, where its action as a sympathetic depressor might have seemed useful on theoretical grounds.

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BLOOD PHOSPHORUS IN HEALTH AND DISEASE: V.—NOTE ON THE CHEMICAL COMPOSITION OF THE RETICULOCYTE.

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The phosphorus compounds of normal human blood can be separated into two classes:

(*a*) Acid-soluble phosphorus compounds, not precipitated when proteins are removed by trichloro-acetic acid, and insoluble in alcohol-ether (3 : 1).

(*b*) Alcohol-ether-soluble phosphorus compounds, precipitated from blood by protein precipitants, and separable from class (*a*) by reason of their solubility in alcohol-ether.

That these two classes were sharply separable from one another was first demonstrated by Bloor (1918), who also showed that their sum was equal,

within experimental error, to the total blood phosphorus determined directly. This is equivalent to showing that compounds of the type of nucleoproteins (or nucleic acid) or other phosphorus-containing proteins, which would be precipitated both by trichloro-acetic acid and by alcohol-ether, do not occur to any appreciable extent in normal human blood. That this is the case has been confirmed repeatedly by F. B. Byrom and myself in the course of investigations on the phosphorus compounds of the blood in health and disease (some aspects of which we have already reported in this Journal [1927, 1928, 1929]), not only for the blood of normal individuals, but also for that of the majority of cases of disease which we have examined. The same absence of nucleic acid phosphorus is demonstrable, as would be expected, when separated red cells are submitted to a similar method of quantitative analysis.

Leucocytes, it is known, contain appreciable amounts of nucleic acid, as do nucleated red cells. But the very small relative quantity of white cells in normal human blood brings their nucleic acid phosphorus, when distributed over the whole-blood figures, within the limits of the experimental error of the method. When the determinations are made on separated red cells, the very small possible leucocyte error may be considered completely eliminated if care is taken to pipette from the centrifuge tube, using a wide-bore paraffined pipette and mouthpiece, only the layer of erythrocytes, thus leaving the greater part of the white cells behind. It may be taken, then, that if in a sample of abnormal blood the total red-cell phosphorus is greater than the sum of the acid-soluble and the lipin phosphorus, the difference represents either nucleic acid or phosphoprotein phosphorus—almost certainly the former, since phosphoproteins have yet to be demonstrated as occurring elsewhere than in milk and in egg-yolk, *i. e.* in the natural foodstuff provided by the maternal organism for the young, growing animal.

The results of some analyses of normal red cells, of leucocytes and of nucleated red cells are given in Table I.

TABLE I.

Type of cell.	Mgm. per 100 c.c. cells.				Per cent. nucleic acid P of total P.
	Total P.	Total acid-soluble P.	Lipin P.	Nucleic acid P	
Normal red cells . . .	69	54	16	−1	0
„ „ . . .	67	51	15·5	0·5	0·8
Leucocytes from case of chronic lymphatic leukæmia	256	82	64	110	43
Red cells of domestic fowl .	309	91	26	192	62

During the past three years, as patients suffering from diseases in which the reticulocyte count * has been increased above normal have become available, determinations have been made of the phosphorus partition in their red cells.

* I am indebted to Dr. F. C. O. Valentine for most of the reticulocyte counts recorded in this paper.

The net result of this work seems to indicate that in human reticulocytosis there is a definite, though not large, quantity of nucleic acid in the blood, the amount being very roughly proportional to the percentage of red cells which stain with cresyl blue. There is also an increase in the organic acid-soluble phosphorus ("ester phosphorus") of the red cells in reticulocytosis.

Method of analysis.—Freshly drawn, oxalated blood (0·15% potassium oxalate as anticoagulant) is centrifuged for 15 minutes at 5000–6000 r.p.m., the plasma removed, and the red cells then slowly drawn up from beneath the leucocyte layer with a paraffined wide-mouthed pipette and mouthpiece, taking care to avoid both the leucocytes and the precipitated calcium oxalate. To the red cells an approximately equal volume of 0·85% NaCl is added, and both are thoroughly mixed together by moving up and down in the pipette. (Unless the red cells are diluted in this way, it is impossible to handle them with any accuracy.) A sample of the mixture is centrifuged forthwith in a high speed hæmatocrit for 15 minutes to determine the relative volume of red cells in the mixture.

For purposes of analysis the mixture of red cells and saline is treated as whole blood, exactly * 0·3 c.c. of the mixture being taken in triplicate for total, 3 c.c. for acid-soluble, and 3 c.c. for lipin P (details of analytical methods may be found in Kay and Byrom's [1927] paper).

The results are summarized in Table II.

Among the possible sources of error which might invalidate this work the more obvious are the following:

(*a*) The possible effect of leucocytes: Particularly in relatively anæmic blood, a serious admixture of leucocytes, known as they are to contain nucleic acid phosphorus, might be responsible for the results. This possibility was minimized by centrifuging at high speed and being careful to take none of the leucocyte layer, although owing to the statement of Key (1921) that the specific gravity of the reticulocytes is slightly less than that of normal erythrocytes, as much as possible of the red-cell layer was removed from beneath the leucocytes. In several instances the leucocyte layer could be, and was, removed mechanically.

(*b*) The possible effect of normoblasts: In the blood with a high reticulocyte count these were looked for, and never exceeded 1 normoblast to 4 white cells. Even if the normoblast were 100% nucleic acid, the effect of such a small admixture with very large excess of non-nucleated red cells would be inappreciable.

According to Key (1921) the mature erythrocyte is not a living cell, and contains neither nucleus nor true cytoplasm; the reticulocyte, on the other hand, is an immature erythrocyte, still containing a remnant of cytoplasm. Davidson and McCrie (1928) believe that the microscopic evidence is in favour of the view that the reticulum is not cytoplasmic but a product of nuclear degeneration. The findings of Harrop (1919), that the oxygen consumption of normal mature human erythrocytes is inappreciable, whilst blood containing abnormal numbers of reticulocytes has an oxygen consumption proportional to

* The 0·3 c.c. pipettes, in which 0·3 c.c. of blood occupies some 18 cm., are made in the laboratory and carefully calibrated for washing out.

the percentage of reticulated cells, agree better, perhaps, with the view of Davidson and McCrie than with that of Key. It is not altogether surprising that the reticulocyte (but not the erythrocyte, the stroma of which, according to Haurowitz and Sládek [1928], contains *no* nucleoprotein) should be found to

TABLE II.

Case number.	Sex.	Diagnosis.	Percentage of reticulocytes.	Hæmatocrit on whole blood.	In mgm. per 100 c.c. red cells* to nearest mgm.				Nucleic acid P. ÷ percentage of reticulocytes.
					Total P.	Total acid-soluble P.	Lipin P.	Nucleic acid P.	
393	M	Normal (H. D. K.) . .	0·5	44·5	69	54	16	−1	...
395	F	„ (P. M. T K.) .	0·8	38·5	70	53	17	0	..
431	M	„ (R. E. H.) . .	0·9	39	67	51	15·5	0·5	...
455	...	Fowl's blood . . .	...	40	309	91	26	192	...
†354	F	Hæmolytic icterus . .	70	19	138	86	21	31	0·4
369	F	Same case, one month later	49	...	129	86	18	25	0·5
362	F	Pernicious anæmia at crest of reticulocyte crisis after liver-feeding	28	...	119	92	17	10	0·4
432	F	Pernicious anæmia before liver-feeding	3·4	12·5	91	72·5	17	1·5	...
		Pernicious anæmia at reticulocyte crisis	24	16·5	110	88	16	6	0·25
357	M	Ditto	23	22	113	89	16	8	0·4
358	F	Splenic anæmia . .	20	25	125	84	27	14	0·7
349	M	Acholuric jaundice . .	16–19	28	100	73·5	16	10·5	0·6
410	M	Pernicious anæmia before liver-feeding	2·2	17	91	75	15	1	...
		Pernicious anæmia after liver-feeding	11	18	100	80	16	4	...
453	M	Pernicious anæmia before treatment	1·4	16	92	76	16	0	...
401	M	Pernicious anæmia recovered on liver treatment	0·7	46	79	60	17	2	...
378	F	Ditto	0·6	42	76	56	17	3	...
462	M	„	0·5	46	53	37	16	0	...
433	M	„	0·4	43	72	57	16	−1	...
491	F	Secondary carcinoma of bones	2	30	96	75	18	3	...
454	M	Aplastic anæmia just before transfusion	2·5	14	89	71	15	3	...
405	M	Splenectomy 3 years ago	1·8	37	81	59	20	2	...
407	M	„ 10 years ago	0·2	51	74	56	17	1	...
406	F	Gaucher's disease . .	2	32	76	55	17	4	...

* As calculated from hæmatocrit value of separated red cells resuspended in 0·85% NaCl.
† Case 354 is the same individual whose blood was studied histologically by Valentine (1928).

contain small quantities of phosphorus compounds similar to those abundantly present in the nucleus, and possibly also in the cytoplasm of living, respiring cells. Even the mature red cell, however, no matter how it behaves cytologically, is not without a chemical taint of nuclear degeneration. Adenine

nucleotide, obtainable as it is by direct hydrolysis of nucleic acid, and which, therefore, may be regarded as a product of nuclear breakdown, is known to occur in the normal mammalian erythrocyte (Hoffman, 1925; Buell and Perkins, 1928), together with other nucleotides (Buell and Perkins, 1928).

Caution would demand some emphasis of the statement that the findings reported in this paper refer only to reticulocytosis in human blood in certain cases of disease. All apparent reticulocyte structures, as Cooke (1928) demonstrates, are not necessarily of similar origin, and the correlation between extent of reticulocytosis and the amount of "nucleic acid P" in the blood may not be true for all types of erythrocytes containing basophilic substances.

SUMMARY.

In normal human blood, the usual methods of analysis show no detectable quantity of nucleic acid phosphorus in the red cells. In cases of disease in which there is a marked increase above normal in the percentage of reticulocytes in the blood, small, but quite definite amounts of nucleic acid phosphorus, roughly proportional in quantity to the extent of the reticulocytosis, have been demonstrated. It may be concluded that, whatever the nature of the reticulum itself, the reticulocytes occurring in such diseases probably contain nucleic acid, and may therefore be looked upon as red cells from which the whole of the nuclear material has not been extruded.

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STUDIES ON THE HÆMOLYSIN PRODUCED BY ATOXIC STRAINS OF *B. TETANI*.

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It has been known for many years that tetanus toxin consists of two parts —tetanospasmin and tetanolysin. If a typical strain of *B. tetani* is grown in broth under anaërobic conditions the lysin is secreted early, reaching its maximum in about 19 hours, while the spasmin does not reach its maximum for 7 to 10 days.

In a previous paper (1929) the isolation of many completely atoxic strains of *B. tetani* was reported, and it was thought to be of interest to test if these strains which had not the power of secreting tetanospasmin could secrete tetanolysin. At the same time several of the toxic strains were also tested. For this experiment the strains, selected at random from a large number of stock cultures, were sown into proteose peptone broth and incubated for 2 days at 37° C. under anaërobic conditions. The cultures were then tested against 0·5 c.c. of a 3% suspension in saline of washed red blood-corpuscles of the sheep, the mixtures being incubated for 2 hours at 37° C. In Table I it will be seen that the atoxic strains produce as potent a hæmolysin as do the toxic strains.

For the titration of the hæmolysin either whole cultures were used or the cultures were first filtered through Seitz filters, it having been found that this type of filter did not tend to reduce the amount of lysin present.

Noguchi (1902) showed that normal serum and also cholesterin prevented the action of tetanolysin while lecithin had no such effect. Lansteiner and Eisler (1905), however, found that lecithin also tended to lessen the action of tetanolysin, though to a considerably less degree than cholesterin. The latter authors also found that an ethereal extract of red blood-corpuscles was as powerful an antihæmolytic agent as was cholesterin and quote Müller's experiments, in which it was found that an alcoholic extract of serum had the same effect. These results would indicate that the inhibiting factor in normal serum is lipoid in nature and probably is the cholesterin of the blood. This antihæmolytic action of cholesterin and serum is not exerted against all bacterial hæmolysins, for Wuth (1923) has shown that normal serum does not inhibit the action of the hæmolysin produced by *B. Welchii*, and it is common knowledge that streptolysin is obtained by growing hæmolytic streptococci in broth containing a large percentage of serum. Recently Gordon and Stansfield (1929) have

TABLE I.

Strain.	Amount of culture in c.cs.											
	1.	0·5.	0·25.	0·1.	0·075.	0·05.	0·025.	0·01.	0·0075.	0·005.	0·0025.	0·001.
19 atoxic .	C	C	C	C	C	C	C	P	P	T	—	—
97 ,, .	C	C	C	C	C	C	AC	P	T	—	—	—
99 ,, .	C	C	C	C	C	C	C	AC	P	T	—	—
20 ,, .	C	C	C	C	C	C	C	C	P	P	—	—
23 ,, .	C	C	C	C	C	C	C	AC	AC	T	—	—
Sheep 2 toxic	C	C	C	C	C	C	C	AC	P	P	T	—
25 toxic .	C	C	C	C	C	C	AC	P	P	T	—	—
44 ,, .	C	C	C	C	C	C	C	AC	P	T	—	—
51 ,, .	C	C	C	C	C	C	C	C	AC	P	—	—
63 ,, .	C	C	C	C	C	C	C	AC	AC	P	—	—

TABLE II.

Strain.	Serum dilutions.										M.H.D. control.	Serum control.
	$1/10^{1}$.	$1/10^{2}$.	$1/10^{3}$.	$1/10^{4}$.	$1/10^{5}$.	$1/10^{6}$.	$1/10^{7}$.	$1/10^{8}$.	$1/10^{9}$.	$1/10^{10}$.		
Sheep 2 toxic	—	—	—	—	—	—	—	—	P	AC	C	—
25 toxic .	—	—	—	—	—	—	—	—	—	P	C	—
99 atoxic .	—	—	—	—	—	—	—	—	P	C	C	—
1 ,, .	—	—	—	—	—	—	—	—	P	AC	C	—
5 toxic .	—	—	—	—	—	—	—	—	AC	C	C	—
19 atoxic .	—	—	—	—	—	—	?	P	AC	C	C	—
97 ,, .	—	—	—	—	—	—	—	T	P	AC	C	—
44 toxic .	—	—	—	—	—	—	—	—	P	AC	C	—
51 ,, .	—	—	—	—	—	—	—	—	P	C	C	—
63 ,, .	—	—	—	—	—	—	—	?	P	AC	C	—

C = Complete hæmolysis. AC = Almost complete hæmolysis. P = Partial hæmolysis.
T = Trace of hæmolysis. — = No hæmolysis.

TABLE III.

Strain.	Cholesterin emulsion dilutions. 1/10¹.	1/10².	1/10³	1/10⁴.	1/10⁵.	1/10⁶.	1/10⁷.	1/10⁸.	M.H.D. control.	Cholesterin control.
Sheep 2 toxic	—	—	—	—	P	P	AC	C	C	—
25 toxic .	—	—	—	—	—	—	AC	C	C	—
99 atoxic .	—	—	—	—	—	ST	P	C	C	—
1 ,, .	—	—	—	—	—	T	AC	C	C	—
5 toxic .	—	—	—	—	—	T	P	AC	C	—
19 atoxic .	—	—	—	—	—	—	T	AC	C	—
97 ,, .	—	—	—	—	—	T	C	C	C	—
44 toxic .	—	—	—	—	T	P	AC	C	C	—
51 ,, .	—	—	—	—	ST	T	P	AC	C	—
63 ,, .	—	—	—	—	T	P	C	C	C	—

TABLE IV.

Strain.	Lecithin emulsion dilutions. 1/10¹.	1/10².	1/10³.	1/10⁴.	1/10⁵.	1/10⁶.	1/10⁷.	1/10⁸.	M.H.D. control.	Lecithin control.
Sheep 2 .	C	C	C	C	C	C	C	C	C	—
25 . .	C	C	C	C	C	C	C	C	C	—
99 . .	C	C	C	C	C	C	C	C	C	—
97 . .	AC	C	C	C	C	C	C	C	C	—

C = Complete hæmolysis. AC = Almost complete hæmolysis. P = Partial hæmolysis.
T = Trace of hæmolysis. — = No hæmolysis. ST = Slight trace.

shown that streptolysin is inhibited by lecithin, while Wuth (1923) showed that small amounts of lecithin definitely lowered the hæmolytic activity of the hæmolysin of *B. welchii*.

To demonstrate the effect of normal serum, cholesterin and lecithin, cultures of *B. tetani* were grown in broth for 2 days and then filtered through Seitz filters. The hæmolytic activity of each filtrate was estimated, the minimal hæmolytic dose being taken as the smallest amount of filtrate producing complete hæmolysis of 0·5 c.c. of a 3% suspension in saline of washed red blood-cells of the sheep in 2 hours at 37° C.

Serum. (*Normal Rabbit.*)

Dilutions of the serum ranging from $1/10^1$ to $1/10^{10}$ were made in saline, and to 0·5 c.c. of each of these dilutions 5 M.H.D. of the filtrates were added, and the mixtures allowed to stand at room temperature for 15 minutes. 0·5 c.c. of the red blood-cell suspension was then added to each tube and the mixtures incubated for 2 hours at 37° C. The results are given in Table II.

Cholesterin.

1 c.c. of a 1% alcoholic solution of cholesterin was added to 9 c.c. of normal saline so as to form a fine emulsion. Dilutions of this emulsion were then made in normal saline and treated as for serum. The results will be found in Table III.

Lecithin.

The effect of lecithin was tested on four strains only, two atoxic and two toxic. A 1% ethereal solution of ovolecithin was made and 1 c.c. added to 9 c.c. of saline. Dilutions of this suspension were treated as before. As a control 0·5 c.c. of the lowest dilution of lecithin (*i. e.* 1 c.c. of the suspension added to 9 c.c. of saline) was tested against 0·5 c.c. red blood-cell suspension. It will be seen from Table IV that the lecithin had no antihæmolytic action.

DISCUSSION.

It will be seen that atoxic strains of *B. tetani* secrete as powerful a lysin as do the fully toxic strains, and that this lysin when tested against various substances behaves in exactly the same manner as the lysin of the toxic strains. It is of interest to note the very powerful antihæmolytic effect of normal serum, which is out of all proportion to the cholesterol content of the serum. This would indicate that tetanolysin can play little, if any, part in the naturally occurring disease, and that an antitetanolysin artificially produced is of little value in immunity. While the experiments reported here have been made with normal rabbit serum, normal human and horse sera have also been tested in a few cases and found to have a similar action. Cholesterin exerts a marked antagonistic action against the lysin while lecithin appears to have no such effect. These actions of serum, cholesterin and lecithin separate tetanolysin from streptolysin and the hæmolysin secreted by *B. welchii*. The criticism

has been made that "atoxic" strains are perhaps not genuine strains of *B. tetani*, but the similarity of the lysins secreted by the two types affords further proof, if further proof were needed, that the atoxic strains are indeed true strains of *B. tetani*.

SUMMARY.

Atoxic strains of *B. tetani* secrete as powerful a hæmolysin as do the toxic strains. Normal serum and cholesterin exert a powerful antagonistic effect against tetanolysin while lecithin has no antihæmolytic power.

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THE GROWTH OF *BR. ABORTUS* IN SEALED TUBES.

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SINCE the time of Bang's (1897) discovery of the organism it has been known that *Brucella abortus*, when first isolated from bovine sources, generally fails to grow on the surface of a solid medium incubated under aërobic conditions. Preisz (1903) observed that growth would occur on an agar slope in a tube closed with sealing-wax, if the air in the tube was displaced with pure oxygen. Smith (1924) has shown, however, that the replacement of the air by oxygen is unnecessary, and that the simple closing of the tube with sealing-wax is sufficient to enable growth to occur. Holth (1911) obtained successful results by impregnating the cotton plugs with melted paraffin. Wilson and Nutt (1926) flamed the cotton-wool plug, pushed it into the tube, and inserted a cork soaked in a melted paraffin-vaseline mixture.

From these observations it is clear that, though *Br. abortus* on first isolation from bovine sources will not grow aërobically, it will grow in tubes that are sealed with melted paraffin or sealing-wax. The early workers assumed that under these conditions there was a diminished oxygen pressure in the tubes, which enabled growth to occur, but the more recent work of Huddleson (1921),

Smith (1924), McAlpine and Slanetz (1928) and others suggests that it is not the lowered oxygen pressure but the increased CO_2 pressure that is of importance.

If it is accepted that free CO_2 in a concentration greater than that present in atmospheric air (0·03%) is necessary for the growth of *Br. abortus*, the problem is to determine why growth should occur in sealed tubes. Both Smith (1924) and Huddleson (Huddleson, Hasley and Torrey, 1927) assume that the reason is because CO_2 is produced by the organisms seeded on to the slope, and as this gradually increases in amount it reaches a concentration sufficient to permit growth to occur. In unsealed tubes the small amount of CO_2 formed would escape, and hence the requisite concentration for growth would not be attained.

This solution of the problem is unsatisfactory. It seems improbable—at least with small seedings—that the simple respiration of the bacteria, in the absence of growth, would produce sufficient CO_2 to raise the concentration to 0·25%, or even to 0·1%, which Smith showed might be sufficient to enable delayed growth to occur. The experiments recorded in the present paper lead to a different solution.

IS GROWTH OF *BR. ABORTUS* IN AIR CONTAINING 5% CO_2 DUE TO ALTERATION IN THE HYDROGEN ION CONCENTRATION OF THE MEDIUM?

Heart-extract agar was prepared. Batch A was brought to a pH of 6·2, Batch B to pH 7·0, and Batch C to pH 7·9. Each medium was delivered in 4 c.c. quantities into $\frac{5}{8}$-in. hard glass tubes; these were autoclaved and sloped in the usual way. A second set of tubes was prepared from each batch containing bromthymol blue or phenol red, the total quantity of dye and

TABLE I.

Incubation.	Time in days.	Batch A pH 6·2. pH of sterile medium.	Batch A pH 6·2. Growth on slopes.	Batch B pH 7·0. pH of sterile medium.	Batch B pH 7·0. Growth on slopes.	Batch C pH 7·9. pH of sterile medium.	Batch C pH 7·9. Growth on slopes.
Aërobically	2	6·2	−	7·0	−	7·8	−
„	6	6·2	−	7·0	−	7·8	−
„	14	6·0	−	6·8	−	7·7	−
„	21	6·0	−	6·8	−	7·7	−
Sealed tubes	2	6·2	−	*6·8 / 6·9	−	7·4 / 7·8	−
„	6	6·2	+++	6·8	+++	7·3 / 7·5	+
„	14	6·0	+++	6·7	+++	7·1 / 7·2	++
„	21	6·0	+++	6·6	+++	6·9 / 7·1	++
5% CO_2	2	6·2	++	6·4 / 6·8	++	6·4 / 7·4	++
„	6	6·2	+++	6·4	+++	6·4 / 6·9	+++
„	14	6·0	+++	6·4	+++	6·4	+++

* The upper figure refers to the pH at the top, the lower figure to that at the bottom of the column of agar.

medium being 4 c.c.; these tubes, after autoclaving, were stood upright, so that the agar set in a solid column. The agar slopes were inoculated with a loopful of a suspension made by adding 4 c.c. of broth to an agar slope culture of a recently isolated bovine strain of *Br. abortus*; the dye-containing tubes were not inoculated. One set of tubes of each batch was then incubated aërobically, another in tubes sealed with paraffined corks (after a preliminary flaming of the cotton-wool plugs), and a third set in a jar containing air, 5% of which had been replaced by CO_2. The results are shown in Table I.

It will be noted that no growth occurred in any of the tubes incubated aërobically, but that in the sealed tubes, and in those incubated in 5% CO_2, the organisms developed freely. In the sealed tubes the growth was slightly less on the medium of pH 7·9, but there is nothing to indicate that a medium on the acid side of neutrality, such as results from incubation in 5% CO_2, is requisite for the development of *Br. abortus*. This finding is in agreement with the conclusions reached by Smith (1924) and by McAlpine and Slanetz (1928).

THE PRODUCTION OF ACID IN UNINOCULATED SEALED TUBES.

Examination of Table I shows that the reaction of all three batches of uninoculated medium incubated aërobically remained practically constant, showing a fall of only 0·1–0·2 pH. In the tubes incubated in 5% CO_2 the reaction rapidly attained a pH of about 6·4, the medium in batches B and C becoming acid from above downwards, consequent on the diffusion of the gas through the agar. In the sealed tubes a similar process of acidification took place, but it was less marked and occurred more slowly.

This progressive acidification of the uninoculated medium in sealed tubes was a striking phenomenon, for which no obvious explanation was forthcoming. The fact that it did not occur when the tubes were incubated aërobically suggested that it must be due to the development of some volatile acid which was retained by the paraffined corks and underwent gradual accumulation. Prof. Raistrick, who kindly discussed this question with us, suggested that in an alkaline medium the agar, which is a complex polysaccharide, might undergo slow hydrolysis with the consequent production of CO_2. To test this we prepared ten different batches of agar, varying in the amount of peptone and phosphate buffer they contained. Glucose was added to some batches, as it was thought that the CO_2 might be in part derived from the muscle-sugar in the heart extract which was used for the preparation of nutrient agar. The reaction of all media was brought to about pH 7·8. One set of tubes was incubated aërobically, while another set was sealed with paraffined corks after preliminary flaming of the cotton-wool plugs. It is unnecessary to reproduce the details of the experiment here; the results were perfectly clear. In the tubes incubated aërobically the reaction remained much the same, never falling more than pH 0·3. In the sealed tubes, on the other hand, progressive acidification occurred in all media, the final reaction after 3 weeks' incubation at 37° C. being about pH 6·8. In order to control this experiment two tubes of M/200 phosphate buffer solution of pH 7·8 were put up. One tube, which was incubated aërobically, had a reaction after 3 weeks' incubation of pH 7·5; the

other tube, which was sealed, had a reaction of pH 6·9. This experiment indicated that the production of acid in sealed tubes occurred independently of the presence of agar or glucose in the medium, and suggested an entirely different source for its origin.

PRODUCTION OF CO_2 BY BURNING COTTON-WOOL PLUGS.

It was observed that if a weak (M/700) phosphate buffer solution of pH 7·8 was used, and the tube containing it was sealed after preliminary flaming of the cotton-wool plug, the reaction fell within a few minutes to pH 6·8 or less, whereas in an unsealed tube it remained constant. This suggested that a comparatively large amount of some volatile acid must be generated during the actual process of sealing. Suspicion fell on the cotton-wool plug. Accordingly 3 c.c. of baryta-water were delivered into clean test-tubes, which were sealed in various ways with plain bark corks, paraffined corks, rubber corks, and with a paraffin-vaseline mixture alone, either with or without preliminary flaming of the cotton-wool plug. Ten minutes later the tubes were shaken and examined. In all tubes in which the cotton-wool plug had been flamed there was a heavy precipitate; in the remaining tubes there was no precipitate. This experiment showed that CO_2 must be given off by the flamed cotton-wool plug.

To test this further, a series of analyses were made of the air inside the test-tubes after flaming of the plugs. $6 \times \frac{3}{4}$-in. test-tubes were used; the plug was cut off flush with the surface, held in the Bunsen burner for 1 to 5 seconds, and pushed for about 1 in. into the tube, which was then closed with a rubber cork having a single short glass delivery tube. This tube was immediately connected by pressure tubing to the burette of a Haldane apparatus, and a specimen of the air withdrawn for analysis. The results are shown in Table II.

TABLE II.

	Method of sealing.	No. of analyses.	CO_2 percentage.
1	Control. Plug not burnt	1	0·05
2	Plug burnt for 1–5 seconds, pushed into tube, and corked immediately	6	0·484—4·50 Mean = 2·144
3	Plug burnt for 1–5 seconds, pushed into tube, and corked 15 seconds later	5	0·297—2·80 Mean = 1·630
4	Plug burnt for 1–5 seconds, pushed into tube, and corked 60 seconds later	3	0·216—1·97 Mean = 1·249

It appears that a considerable amount of CO_2 is generated by a flamed cotton-wool plug. The actual amount produced seems to depend largely on the time during which the plug is held in the flame; the highest figures were generally obtained with plugs flamed for 5 seconds. The conclusions to be drawn are that if the plug is flamed for 2 to 3 seconds, and the cork inserted about 15 seconds later, the percentage of CO_2 in the tube may be expected to be about 1 to 2%.

These analyses were conducted on tubes having a free air-space of about 20 c.c. In tubes containing 6 c.c. of agar the free air-space would naturally be less, and in such tubes the percentage of CO_2 might be expected to be of the order of 1·5 to 3%.

THE PRODUCTION OF CO_2 BY MELTED PARAFFIN AND BY SEALING-WAX.

Further work on the composition of air inside tubes sealed in various ways led to the suspicion that small quantities of CO_2 might be given off by a melted paraffin-vaseline mixture (*partes æquales*). A 6 × ¾-in. test-tube was taken, and the bottom was cut off. One end was fitted with a rubber cork having a single short glass delivery tube connected to a piece of pressure tubing closed with a screw-clip. A loose unflamed cotton-wool plug was pushed into the other end, and a bark cork, which had been dipped in a melted paraffin-vaseline mixture, heated to the vaporization point, was inserted. After the paraffin had set, a sample of air inside the tube was withdrawn through the delivery tube at the other end, and analyzed in the Haldane. The results showed that the CO_2 in such tubes varied from 0·124 to 0·220%, compared with a value of 0·09%* in tubes fitted with non-paraffined corks.

Similar analyses were made of the air in tubes that had been closed at one end with sealing-wax. In this case a thin layer of asbestos wool was laid on top of the cotton-wool plug to prevent the latter being burnt, and the tube was then closed with red sealing-wax. Three consecutive analyses of the air in these tubes gave figures of 0·32, 0·32 and 0·33% CO_2, as compared with 0·03% in unsealed tubes.

PRODUCTION OF CO_2 BY RED RUBBER CORKS.

It was noticed that weak (M/700) alkaline phosphate buffer solution contained in tubes sealed with plain red rubber corks became acid after incubation at 37° C. for 2 or 3 weeks. To ascertain whether CO_2 was produced under these conditions, large boiling tubes were taken, and sealed with rubber corks having a single glass delivery tube closed at the outer end. The tubes were placed in the incubator, and one was withdrawn every few days for examination. The glass tube was broken off and immediately attached to a Haldane apparatus. The results were as follows: In 12 tubes examined after 8 to 28 days' incubation, the CO_2 content varied from a minimum of 0·042% to a maximum of 0·710%; the average value after 3 weeks was 0·171%. Owing to the crudeness of the method employed, this figure is probably somewhat below the real value. Since the free air content of the tubes was about 170 c.c., it may be calculated that 0·232 c.c. of CO_2 must on the average have been produced by each cork. If the CO_2 is given off by the narrow end of the cork, then it may be deduced that in a 6 × ⅝-in. test-tube containing 4 c.c. of agar and having a free air-space of 16 c.c., a rubber cork with a diameter at the narrow end of 1·3 cm. would raise the CO_2 content to about 0·27%.

GROWTH OF *BR. ABORTUS* IN TUBES SEALED IN DIFFERENT WAYS.

From the foregoing experiments it appears that CO_2 is given off in moderately large quantities by burning cotton-wool plugs, and in smaller quantities by melted paraffin, by sealing-wax and by red rubber corks.

Experiments made to determine the minimum percentage of CO_2 necessary for the growth of the particular strain used in this work showed that in an

* It has been found that, when the windows are closed and numerous gas points are burning, the CO_2 in the laboratory air may reach a figure of 0·1%.

atmosphere containing 0·5% CO_2 slight growth occurred in 5 days, whereas in the presence of 1% CO_2 abundant growth occurred in 2 days.

If, as seems probable, the growth of *Br. abortus* in sealed tubes is to be accounted for by the production of CO_2 by the seal itself and not by the respiring organisms, it should be possible to demonstrate a parallelism between the nature of the seal and the degree of bacterial growth.

To test this an agar slope culture of *Br. abortus*, which had been grown in 5% CO_2, was washed off with 4 c.c. of broth, and a small loopful of the suspension was inoculated on to a number of agar slopes of pH 8·2. A similar number of uninoculated tubes were used for the colorimetric determination of the H-ion concentration; a suitable amount of phenol red was added to the melted agar, which was allowed to set in a solid column. (It was in order to follow the progressive acidification of the medium with a single indicator that the reaction was adjusted to such a high pH.) Both sets of tubes were sealed and incubated at 37° C. The results are given in Table III.

TABLE III.

	pH of sterile agar.			Growth in inoculated tubes.
	4 days.	12 days.	21 days.	
Control in 5% CO_2	6·4	6·4	6·4	+++ 2 days.
Aërobic. Plug not burnt	8·2	8·1	8·0	− 21 days.
„ „ burnt	8·1	8·1	8·0	− 21 days.
Sealed. Bark cork, plug not burnt	8·2	8·1	8·0	− 21 days.
„ „ „ burnt	7·7 8·2	7·5 7·8	7·4 7·6	± 7 days, ++ 12 days.
„ Paraffined cork, plug not burnt	8·1	8·0	7·8	++ 12 days.
„ „ plug burnt	7·3 8·1	7·0 7·4	7·0 7·1	+++ 4 days.
„ Rubber cork, plug not burnt	8·2	8·0	7·0 7·2	± 12 days, ++ 21 days.
„ „ plug burnt	7·4 8·1	7·2 7·6	7·1 7·3	+++ 4 days.
„ Paraffin-vaseline, plug not burnt	8·2	8·1	8·1	+ 21 days.
„ „ plug burnt	7·6 8·1	7·5 7·7	7·4 7·6	+ 4 days, +++ 7 days.
„ Sealing-wax, plug not burnt	8·0	7·8	7·7	± 21 days.
„ „ plug burnt	7·6 7·9	7·6	7·5	+ 16 days, ++ 21 days

It will be noticed that, apart from the 5% CO_2 control, in which abundant growth was present after 2 days, growth occurred most rapidly and luxuriantly in those tubes which were sealed with paraffined or rubber corks, after preliminary flaming of the cotton-wool plug. It was in these tubes likewise that the H-ion concentration showed the most rapid increase.

It is surprising that growth in the tubes closed with sealing-wax was not more abundant. The tests were repeated on three or four occasions, and the results with these tubes were irregular. For example, in one experiment in which the plug was flamed, abundant growth occurred in 5 days, and in another

experiment not till 14 days. In two experiments in which the plug was not flamed no growth occurred even after 21 days, while in two other experiments abundant growth was present after 7 days. These irregularities may perhaps have been due to small undetected leaks in the sealing-wax, which allowed the CO_2 formed during the process of sealing to escape. From a practical point of view sealing-wax appears to be less reliable than paraffined or rubber corks.

It is interesting to note in Table III the marked fall in pH of the tube that was fitted with a rubber cork without preliminary flaming of the plug. The fall occurred slowly, but was progressive, probably indicating the continuous evolution of CO_2 by the cork. In this particular case the process of acidification was most active during the third week; in another experiment the pH dropped to pH 7·7 by the twelfth day, and to 7·6 by the twenty-first day.

These results, which, with the exceptions noted above, may be taken as representative of similar experiments made on three or four other occasions, are in consonance with the view that the growth of *Br. abortus* in sealed tubes is related to the amount of CO_2 produced by the seal.

SUMMARY AND CONCLUSIONS.

1. Recently isolated strains of the bovine type of *Br. abortus*, though unable to grow aërobically in open tubes, develop satisfactorily in the presence of air if the tubes are suitably sealed.

2. Evidence is brought to show that the growth in sealed tubes is due to the presence of CO_2 given off by the seal.

3. It is shown that CO_2 is produced by flamed cotton-wool plugs, and to a less extent by melted paraffin, by sealing-wax, and by plain red rubber corks.

4. In tubes sealed with paraffined corks, after preliminary flaming of the cotton-wool plug, the CO_2 content of the air over the medium is about 1 to 3%.

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THE PREPARATION OF ANTIGENIC SPECIFIC SUBSTANCE FROM STREPTOCOCCI AND PNEUMOCOCCI (TYPE I).

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In the study of infection and immunity the agglutination reaction has been generally employed to show certain important differences between bacteria of the same species. By this means various strains can be classified in serological types. The reaction depends on the presence in the bacterium of a "specific substance" which in its antigenic capacity provokes the formation of the type-specific agglutinin and precipitin. Further, the presence of the type-specific substance has been shown to be intimately related to the virulence—actual or potential—of pneumococci by Stryker (1916), Griffith (1923) and others, while Todd and Lancefield (1928) have demonstrated a similar relation in the case of hæmolytic streptococci.

Various methods have been employed for the extraction of the type-specific substance from pneumococci and streptococci, but none have succeeded in obtaining this substance in an active antigenic condition. While the solutions obtained give type-specific precipitin reactions with immune sera (prepared by injecting whole bacteria), they are incapable of provoking type-specific antibody formation on injection in animals.

In a former communication it was shown that from bacilli of the typhoid group and from staphylococci it is possible to prepare solutions which contain the specific substances in an active antigenic state. A single intravenous injection of such a solution in the rabbit is followed by the appearance of specific agglutinin and precipitin in its serum. While solutions of the typhoid antigens are stable, the antigenic specific substance obtained from staphylococci loses its antigenic power unless removed from the action of the bacterial enzymes.

This research has been continued with the object of obtaining type-specific substances from streptococci and pneumococci in an active antigenic condition. In this work a large number of preparations were made and tested as follows:

(*a*) A single dose of a clear bacterial extract was injected intravenously in a rabbit.

(*b*) The rabbit was bled 6 or 7 days later, and the serum tested for its agglutinating power, with the usual controls, against two strains of the bacterial species.

For investigation the following streptococci were employed: *S. hæmolyticus* (2 strains); *S. viridans* (2 strains) and 2 strains of an indifferent variety. All

were derived from human sources and were pathogenic to mice. A pneumococcus of Type I was used, and its virulence maintained by regular passage through mice. Degraded and Type IV pneumococci were employed as controls.

Stock cultures of these organisms were kept in anaërobic meat tubes at room temperature. For purposes of extraction and for agglutination tests the cocci were grown overnight in trypsinized broth with 2% serum at 37° C. and the medium enriched next morning by the addition of glucose broth. After 2 to 3 hours the cocci were collected by the centrifuge, washed in saline and resuspended in saline for agglutination tests.

Agglutination Technique.

The rabbits employed were bled from the marginal ear vein into tubes which were kept in the ice-chest till next day, when the serum was removed from the clot.

Trial agglutination tests were performed at different temperatures: room (15° C.), at 37° C., at 45° C. and at 55° C. for 2 hours, with reacting sera and organisms.

It was found that the optimum temperature for the test varied with the age of the serum. Fresh serum reacted in higher titre at room temperature than at higher temperatures, but took longer than 2 hours to show the final result. At 45° C. the readings were generally sharper with hæmolytic streptococci, but with other types agglutination was delayed and imperfect. Previous inactivation of the fresh serum made very little difference to these reactions. 55° C. was found unsuitable as likely to induce auto-agglutination.

After the serum had been stored some days in the ice-chest its reactivity at different temperatures was modified. The serum now showed better agglutination at 45° C. than at lower temperatures, and a final reading was possible in 2 hours.

The following experiment is an example of such modification:

Rabbit Injected with an Extract of Streptococcus viridans; *Serum collected 6 days later.*

Dilution.	Tested next day.		Re-tested after storage 1 week.	
	At 15° C.	At 45° C.	At 15° C.	At 45° C.
1:1	++	Slight	++	++
1:3	+	0	+	+
1:7	+	0	+	+
1:15	Slight	0	Slight	+
1:31	0	0	0	0
Control	0	0	0	0

CONTENT OF TYPE-SPECIFIC ANTIGEN IN DIFFERENT BACTERIA.

To obtain a specific response to a single intravenous injection of a bacterial extract it is necessary that the bacteria used should contain a certain amount of type-specific antigen. Both typhoid bacilli and staphylococci are relatively rich in these substances; a single injection of a vaccine (or of an active solution)

of either organism is sufficient to provoke a strong specific response in a rabbit. The serum taken a week later will generally agglutinate to a titre of 1 : 1000 and give a definite precipitin reaction with solutions of the bacteria. But with the organisms now under investigation—pneumococci and streptococci—the conditions are quite different.

Pneumococci.—Using virulent pneumococci Type I, a single dose of killed cocci will have no effect, or at most yield a serum which when undiluted will barely agglutinate the pneumococci. Yet solutions of these pneumococci tested against type-specific serum show a fair content of specific substance. It requires a series of injections of Type I pneumococci to produce a specific antiserum of even moderate titre; and it has been shown that certain Type III pneumococci are incapable of provoking type-specific antibody formation, although they contain type-specific substance (Tillett, 1927).

If a rabbit be given two or three doses of Type I pneumococcus vaccine, a further serial dose will definitely raise the agglutinating titre of its serum, although an equal dose is without such effect on a normal rabbit.

These considerations suggest that the specific substance contained in the capsules of virulent pneumococci is present in a non-antigenic state, but that the cocci contain a very small amount of active antigenic specific substance. When some immunity is already present, injected pneumococci are disposed of more readily, and antigenic specific substance is set free under conditions more favourable for provoking antibody formation. If the antigenic specific substance could be obtained from pneumococci *in vitro*, its injection should act better than a single dose of the cocci (bacterial vaccine).

Streptococci.—Measured by the ability of a single dose of killed bacteria to provoke the formation of type-specific antibody, the pathogenic streptococci exhibit wide differences, irrespective of their nature (hæmolytic, viridans) or mode of culture. Not only do different strains thus vary, but each strain seems liable to produce variants which may fail to develop type-specific antigen. Hence in the course of this research it was necessary to secure strains that contained sufficient type-specific antigen, and to test their stability from time to time by the effect of injections of the whole killed cocci.

A further difficulty with streptococci, compared with other organisms, lies in the actual extraction of the type-specific substance. By means of fractional solution by successive applications of dilute antiformin (as described in a former communication), it was found that the type-specific substance might only be liberated after disintegration of the streptococci was advanced. In the case of one hæmolytic strain the specific substance was only obtained in the last fraction, after practically all the protein had been removed.

METHODS OF EXTRACTION ADOPTED.

Since extraction of these bacteria with antiformin or by Porges' method (N/20 HCl at 100°) has been found to give solutions of the type-specific substances only in a non-antigenic condition, it was necessary to use methods which secured good disintegration and solution without the employment of ordinary chemical reagents or high temperatures. Further, the process should not involve the exposure of liberated antigen to the action of bacterial enzymes for any length of time, since this might result in loss of antigenic activity.

Young Gram-positive cocci, grown as already described, were collected and washed in saline by means of the centrifuge. They were again spun, resuspended in water, and at once mixed with an equal volume of alcohol. This suspension of killed cocci was centrifuged and the supernatant fluid discarded, leaving only a small amount above the deposit of cocci. In these few drops the deposit was resuspended by means of a pipette, and spread on sterile glass slides, which were then dried in the incubator. The slides were stored in closed vessels over $CaCl_2$ until required.

To prepare solutions the films of dried cocci were scraped off the slides and ground with successive additions of liquid in a sterile Hayden's mortar, the resulting suspension being transferred in similar amounts to a collecting tube. This process of grinding was productive of a decided alkaline change which aided solution.

After the short time allowed for extraction (as described below), the suspension was centrifuged and the supernatant extract removed and neutralized with a trace of weak acetic acid. It was rarely possible to get a perfectly clear extract by simple centrifugalization. However, it was found that the addition of purified kaolin (kaylene) during or in the last stages of grinding greatly facilitated clearing on centrifuging, probably through the mutual precipitation of colloids. To avoid adsorption of antigen only a small amount of kaylene was used, about equal in bulk to the cocci. Colloidal aluminium hydroxide (alocol) acted similarly to kaylene.

For the extraction of the ground cocci various liquids were tried:

(*a*) Cold saline was successful with certain streptococci comparatively rich in specific antigen. Water gave very similar results, the solutions having salt added before injection.

(*b*) Hot saline (or water), maintaining a temperature of 80° C. for 15 or 20 minutes, or 60° C. for twice this time gave much better results.

Examples:

Streptococcus hæmolyticus.—Alcoholized and dried. Equal amounts ground with kaylene in water (*a*) at room temperature, (*b*) kept at 80° C. for 20 minutes. Both centrifuged clear, neutralized, and saline added to render isotonic. Injected intravenously in 2 rabbits, which were bled 6 days later.

	Agglutination titre.
(*a*) Cold extract	1: 8
(*b*) Hot extract	1:16

Streptococcus viridans.—Alcoholized and dried. Equal amounts ground in saline, kaylene added at end, (*a*) at room temperature, (*b*) at 60° C. for 30 minutes. Both centrifuged clear, neutralized and injected as in above example.

	Agglutination titre.
(*a*) Cold extract	1: 8
(*b*) Hot extract	1:64

Pneumococcus (Type 1). Alcoholized and dried. Equal amounts ground in saline with alcohol (*a*) at room temperature, (*b*) at 60° C. for 35 minutes, (*c*) at 80° C. for 20 minutes. All centrifuged clear and neutralized. Injected intravenously in 3 rabbits which were bled 6 days later.

	Agglutination titre.
(*a*) Cold extract	1:4
(*b*) At 60° C.	0
(*c*) At 80° C.	1:4

In no case was extraction at 100° C. successful.

(*c*) Bile salt: Sodium taurocholate ($\frac{1}{2}$ to 1% solution) was found a good solvent, provided heat was used for a sufficient time. But this reagent had the drawback that the extract could not be properly cleared by the centrifuge, since a fine colloidal suspension resulted. When this centrifuged extract was treated with two volumes of acetone, a flocculent precipitate appeared within a few minutes, especially if placed in the ice-chest. This precipitate was quickly collected, dried and taken up in saline, and could be centrifuged practically clear. Experiments showed that a fair proportion of the active antigen might be lost through adsorption to protein rendered insoluble by the coagulating action of the acetone, but in spite of this loss, the taurocholate process gave good results.

Examples:

Streptococcus viridans.—Alcoholized and dried. Equal quantities ground rapidly and extracted with 0·6% taurocholate at 60° C. for (*a*) 15 minutes, (*b*) 40 minutes. Suspensions centrifuged and the supernatant fluids treated with 2 vols. acetone. Precipitates dried, dissolved in formol-saline and centrifuged clear. Solutions injected intravenously in 2 rabbits. Serum taken 6 days later.

	Agglutination titre.
(*a*) 15 minutes' extract	0
(*b*) 40 minutes' extract	1:64

Streptococcus.—Indifferent from sputum. Similar experiment.

	Agglutination titre.
(*a*) 20 minutes' extract	1: 8
(*b*) 40 minutes' extract	1:32

Streptococcus hæmolyticus.—Similar experiment.

	Agglutination titre.
(*a*) 15 minutes' extract at 60° C.	0
(*b*) 45 minutes' extract at 60° C.	1:16
(*c*) 20 minutes' extract at 80° C.	1:32

Experiments on similar lines demonstrated that no antigenic specific substance was obtained when extraction with taurocholate was performed at room temperature or at 100°C. for 15 minutes.

It was necessary that the acetone precipitate should be obtained and collected as quickly as possible. The longer the precipitate was left in acetone, the more insoluble it proved, owing to the coagulating effect on the protein.

This taurocholate process did not succeed with pneumococci. Further experiments with streptococci showed that drying and grinding of the bacteria were unnecessary in this process.

Example:

Streptococcus viridans.—A suspension of cocci from a young culture was alcoholized, divided in two equal portions and centrifuged. The cocci in the one tube were dried, in the other washed in water and centrifuged. Dried cocci were ground and extracted with 0·6% taurocholate at 60° C. for 40 minutes; the washed cocci were similarly extracted. Both suspensions centrifuged and the supernatant fluid treated with 1½ volumes of acetone. The precipitates dried, dissolved in saline and centrifuged. These solutions injected intravenously in 2 rabbits which were bled 6 days later.

	Agglutination titre.
(*a*) Extract of dried and ground cocci	1:64
(*b*) Extract of moist intact cocci	1:64

MIXED BACTERIAL DIGESTS.

It was accidentally discovered that when streptococci were added to a culture of pneumococci and the mixture incubated, some solution of the streptococci accompanied autolysis of the pneumococci, and specific substance from the streptococci was liberated in an antigenic condition. Similarly an admixture of dried pneumococci increased the yield of antigen when streptococci were ground in saline or water at room temperature. Degraded pneumococci acted better than virulent Type I cocci; and staphylococci that had been deprived of their specific antigen (by keeping a suspension of killed staphylococci until they were Gram-negative) were superior to pneumococci in this respect.

Examples:

Streptococcus hæmolyticus.—Alcoholized and dried. Ground (*a*) alone, (*b*) with Gram-negative staphylococci. Suspensions centrifuged clear with alocol added. Injected intravenously in 2 rabbits which were bled 6 days later.

	Agglutination titre.
(*a*) Simple extract	1: 8
(*b*) Mixed extract	1:32

Streptococcus viridans.—Alcoholized and dried. Equal quantities ground in saline with (*a*) virulent pneumococci, (*b*) degraded pneumococci, also alcoholized and dried. Suspensions centrifuged clear and injected intravenously in 2 rabbits. Sera collected 7 days later.

	Agglutination titre.
(*a*) Using virulent pneumococci	1: 16
(*b*) Using degraded pneumococci	1:128

Streptococcus viridans (another variety).—Similar experiment.

	Agglutination titre.
(*a*) Using degraded pneumococci	1: 4
(*b*) Using Gram-negative staphylococci	1:16

EXTRACTION WITH CHEMICAL SOLVENTS.

Weak alkali (NaOH) and weak acid (HCl) can be used for the extraction of antigenic specific substance from bacteria. These agents act much better on suspensions of intact killed bacteria than on dried and ground organisms. But the use of these chemicals has a serious disadvantage owing to their action on the bacterial protein. Alkali, if its action be prolonged beyond a short time, or if heat be applied, forms a compound with the protein dissolved. This altered protein is precipitated on neutralization of the extract and carries down most or all of the antigen by adsorption, thus leading to great loss.

Similarly with acid, thought it has less solvent power on the bacterial protein, it renders it precipitable on neutralization. This difficulty, however, may be avoided by removal of the antigen from the acid extract, using a special method of adsorption that will be described later.

The following experiment illustrates this extraction with acid:

Streptococcus viridans.—Killed by alcohol and half the suspension dried; the other half washed in water. (*a*) The dried cocci ground in N/20 HCl and kept at 60° C. for 40 minutes. (*b*) The washed cocci treated with N/20 HCl at 60° C. for 40 minutes. Both extracts centrifuged clear and the antigen removed as an adsorbate. Injected in 2 rabbits which were bled 8 days later.

	Agglutination titre.
(*a*) Acid extract of dried and ground cocci	1: 2
(*b*) Acid extract of intact cocci	1:256

Pneumococcus (Type I).—Washed and extracted with N/20 HCl at 60° C. for 40 minutes. The extract centrifuged clear and the antigen removed as an adsorbate. Injected intravenously in a rabbit which was bled 6 days later.

	Agglutination titre.
Acid extract	1:2

Comparative experiments show that much the same yield of type-specific antigen was obtained by the three usual processes used, viz. extraction with hot saline, bile salt or acid.

The quantity of bacteria used for extraction was regulated by the amount of specific antigen contained, as determined by the response of a rabbit to injection of the bacterial intact bodies (vaccine). With the richer strains of *S. viridans* employed, half the cocci obtained from a 20-hour culture was sufficient; with other streptococci a whole culture was used. To obtain antigenic specific substance from the pneumococci it was necessary to use the cocci from two or three cultures for each extract.

The extracts described did not give rise to toxic symptoms on injection in rabbits.

PROPERTIES OF THE TYPE-SPECIFIC ANTIGEN.

All observers have found that the type-specific substance as obtained in a non-antigenic condition is a remarkably stable body, resisting heat and chemical agents. The actively antigenic solutions obtained by the methods above described were therefore tested by injection to determine the stability of the antigenic group on which their activity depended.

(*a*) Heat: Their activity in provoking antibody response was lost after heating to 100° C. for 15 minutes. The type-specific antigens were therefore heat-labile.

(*b*) Their activity was diminished when kept in solutions of alkaline pH.

Example:

Streptococcus viridans.—Alcoholized, dried. Ground in water with alocol. Centrifuged clear and saline added to make isotonic. Not neutralized; pH = 9. Half injected same day; other half after 10 days in ice-chest.

	Agglutination titre.
(1) Control	1 : 32
(2) Kept 10 days in alkaline solution	1 : 4

Exposure to weak alkali for a short time does not injure the antigen. An active solution of neutral pH was treated with NaOH at a concentration of 1 : 2000 for 5 minutes. and then neutralized. This half of the solution showed no loss of activity as compared with control untreated half on test by injection.

(*c*) Antigenic specific substance suffers loss of activity when exposed to the action of enzymes liberated in old broth cultures of the organism.

Example:

Streptococcus viridans.—Alcoholized and dried. Ground in hot saline with alocol and kept at 60° C. for 30 minutes. Centrifuged clear and neutralized. Divided in three equal parts: (1) control, (2) heated to 100° C. for 15 minutes, (3) diluted with clear supernatant broth from old culture. All kept 2 days in ice-chest and then injected in three rabbits.

	Agglutination titre.
(1) Control	1 : 1024
(2) Heated	0
(3) Treated with old culture	1 : 64

This inactivation of antigenic specific substance appears to be due to the action of a special enzyme in the bacterium which is liberated in culture media. It has already been shown that streptococci may be disintegrated by the enzymes of pneumococci and staphylococci, and that the specific substance then set free from the streptococci is not inactivated in the process. The

following experiment shows that the particular enzyme contained in or secreted by a streptococcus which is responsible for inactivation of the specific substance may be type-specific too:

Streptococcus viridans.—Alcoholized and dried. Ground in hot saline (then kaylene added) and kept at 60° C. for 45 minutes. Centrifuged clear and neutralized. Divided in two equal parts and added to equal volumes of supernatant glucose broth from (*a*) an old culture of the same strain, (*b*) an old culture of a different strain of *S. viridans*. These two preparations were kept 3 days at room temperature; then one volume of acetone was added to each, and the precipitates re-dissolved in saline and centrifuged clear. Injected intravenously in two rabbits, which were bled 8 days later.

	Agglutination titre.
(*a*) Extract treated with culture fluid of same organism	1: 8
(*b*) Extract treated with culture fluid from another strain	1:512

(*d*) Solutions of antigenic type-specific substance retained their activity well if kept slightly acid at pH 6·5. Carbol-saline was used as a preservative in preference to formol-saline, since the latter had a slow deteriorative action on the antigenic group.

Example:

Streptococcus viridans.—Alcoholized and dried. Ground in cold water, kaylene added after grinding. Centrifuged clear; neutralized. Divided in three equal parts: (1) control, saline added, (2) formol-saline added to give strength 1/2000, (3) carbol-saline added to give strength 1/300. Solutions (2) and (3) kept at room temperature for 15 days before injection.

	Agglutination titre.
(1) Control	1:32
(2) Formolized	1: 8
(3) Carbolized	1:32

Streptococcus hæmolyticus.—Alcoholized and dried. Ground in 6% taurocholate and kept at 60° C. for 45 minutes. Centrifuged. Supernatant divided in two halves and each precipitated with 2 volumes of acetone. One precipitate extracted with saline, the other with formol-saline (1/2000). Both centrifuged clear and kept a week before injection.

	Agglutination titre.
(*a*) Saline	1:128
(*b*) Formol-saline	1: 64

It has been mentioned that if these bacterial extracts be kept alkaline, there is a gradual loss of their antigenic property. This loss is accelerated by exposure to body temperature, even for so short a time as 1 hour. If formol be added in these conditions the antigen is rapidly destroyed.

Example:

Streptococcus viridans.—Alcoholized and dried. Ground and extracted with saline at 60° C. for 45 minutes, kaylene being added. Centrifuged clear and divided in three equal parts: (*a*) kept at pH 6·5 at room temperature, (*b*) kept at pH 8·5 for 1 hour at 37° C., (*c*) kept at pH 8·5 for 1 hour at 37° C. with the addition of formol (= 1/2000 strength).

Solutions (*b*) and (*c*) then rendered just acid (pH 6·5), and all three injected intravenously in 3 rabbits which were bled 6 days later.

	Agglutination titre.
(*a*) Extract kept acid (at 15° C.)	1:128
(*b*) Extract kept alkaline 1 hour (at 37° C.)	1: 32
(*c*) Extract kept alkaline 1 hour (at 37° C.) with formol added	1: 2

(*e*) In its relation to protein, the antigenic specific substance did not appear to differ from the substance in its non-antigenic condition.

From certain streptococci which more readily yielded active antigen on grinding in cold saline, it was possible to obtain an active solution which contained little or no protein. The protein (if present) was precipitated with acid, picric being found better than acetic for this purpose, and the antigen recovered from the supernatant fluid by percipitation with alcohol or acetone (3 volumes).

In solutions made with the aid of heat, bacterial protein was usually present in sufficient amount to carry down almost all the active antigen by adsorption when precipitated with acid. To obtain the antigen apart from the bacterial protein by this method of first precipitating the protein involves the use of large quantities of material and much loss of antigen. A simple method of avoiding loss is to take out the active antigen from the bacterial extract, leaving the bacterial protein in solution. For this purpose advantage was taken of the ease with which the antigen is adsorbed to any protein on precipitation.

To the clear bacterial extract an equal volume of a saturated solution of ammonium sulphate was added and mixed: this produces no precipitate. A few drops of non-immune serum were added and mixed: the resulting precipitate of serum globulin was collected and redissolved in saline. This product was found to possess the full antigenic power of the original bacterial extract. Since a solution of ammonium sulphate has an acid reaction, this method was used to remove the type-specific antigen from bacterial solutions made by extraction with acid.

SUMMARY.

Various methods are described of extracting streptococci to obtain solutions which contain the type-specific substance in an antigenic state, as shown by the results of injections in rabbits. These methods are in general also applicable to pneumococci (Type I).

For the purposes of extraction it is generally (but not always) desirable to employ heat. The temperature used must be regulated since the antigens are heat-labile: extraction at 100° C. is destructive.

The antigenic property of these active extracts is destroyed by agents which do not affect the reactivity of the type-specific substance towards immune serum. In other words, antigenic specific substance possesses two affinities of a different stability, and contains two groups:

(1) A stable portion (ordinary specific substance) which reacts with immune serum (haptophore group).

(2) An unstable antigenic portion which provokes the formation of antibody (toxophore group).

The differences between these groups in stability may be tabulated thus:

Agent.	(1) Serum-reacting.	(2) Antibody-forming.
Heat (100° C.)	Resistant	Destroyed
Antiformin	,,	,,
Acid	,,	Resistant
Formol	,,	Weakened
Bacterial enzyme	,,	Destroyed or split off.

The nature of this antigenic group is conjectural; it resembles an enzyme in some properties.

It is possible to obtain antigenic type-specific substance apart from ordinary bacterial protien, either by removal of the protein from the extract, or by adsorption of the substance to a foreign added material.

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A STUDY OF THE PHOSPHORUS DISTRIBUTION IN BACTERIAL CULTURES.

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THE fact that phosphorus in some form or other is important for the growth of bacteria has long been known, but the distribution of phosphorus in its various combinations in bacterial cultures has not been studied quantitatively to any appreciable extent.

Raulin (1869) was one of the first to point out that the presence of phosphate in a medium, though small in amount, was necessary for the growth of *Aspergillus niger*.

Whilst there are numerous references in the literature to the qualitative use of phosphorus compounds in nutrient media, the most important contribution to the quantitative aspect of the subject is that of Stoklasa (1911), who published data of the distribution of organic and inorganic phosphorus in synthetic media inoculated with soil bacteria and others. In his experiments with lecithin, artificial media were made up containing various inorganic salts and 0·1% lecithin. After inoculation with *B. mycoides*, *B. subtilis* and *B. proteus vulgaris*, the media were incubated at 28–30° C. in the dark for 60 days, and then it was found that 64, 68 and 66% respectively of the phosphorus of the lecithin was converted into inorganic phosphate (orthophosphoric acid).

The dry bacteria themselves contained the following percentages of phosphorus (as P_2O_5): mycoides, 3·62%; subtilis, 4·33%; proteus, 3·70%. These amounts, which are assumed by Stoklasa to be present as nucleoprotein, correspond to approximately 39, 37 and 39% of the phosphorus originally present in the medium as lecithin. These, together with the figures obtained for the inorganic phosphate in the medium, give totals of 103, 105 and 106% respectively of the amounts of phosphorus originally present in the medium as lecithin. The values are probably within the limits of experimental error if we assume that all of the lecithin had been hydrolyzed, but apparently no attempt was made by Stoklasa to determine whether any unchanged lecithin remained in the medium after growth. These results are interesting in so far as they show a very considerable hydrolysis of lecithin by these three organisms under the conditions employed.

In a further series of experiments Stoklasa replaced the lecithin by an equal amount of nucleic acid, and he found that in a medium containing no other nitrogenous foodstuff (such as ammonium sulphate) it was much less effective in promoting the growth of the same three bacteria than was lecithin.

However, on adding glucose and ammonium sulphate to the medium and incubating at 28–30° C. in the dark for 20 days, a much better growth of bacteria occurred with a corresponding increase in the amount of inorganic phosphate liberated, but even then the percentage of inorganic phosphate was less than that produced in the experiments in which lecithin was used.

The different media which are commonly used for bacterial cultures contain phosphorus in at least three forms. These are (1) inorganic phosphate, (2) phospholipins, and (3) organic acid-soluble phosphorus compounds. It appeared of interest to us to investigate the changes occurring in these fractions in a quantitative way, using different organisms. Whole cultures have been used in the majority of cases, and unless otherwise stated the results given were obtained with 48-hour cultures.

EXPERIMENTAL.

The technique employed in the various experiments has been as follows, all values being given in terms of phosphorus (not P_2O_5):

Total phosphorus.—0·5 c.c. was oxidized by 1 c.c. of a nitric-sulphuric acid mixture (conc. acids 2 : 1) and finally heating with a few drops of Merck's perhydrol. The sulphuric-phosphoric ester was hydrolyzed by boiling for a few minutes with water, and the determinations carried out colorimetrically by the Briggs modification (1922) of the Bell and Doisy method (1920).

Inorganic phosphate (*orthophosphoric acid*).—10 c.c. of the medium were pipetted into a small conical flask, and 2 c.c. 25% trichloracetic acid added. After standing half to two hours the contents were filtered, and determinations made on 1 c.c. of the filtrate by means of Bell and Doisy's original method. In the acid solution used in Briggs's method, a permanent precipitate was formed by some constituent of the original bouillon not precipitable by the trichloracetic acid, whereas this precipitate redissolves in the alkaline solution used by Bell and Doisy.

Lipin phosphorus.—5 c.c. of the medium were pipetted into about 85 c.c. alcohol-ether mixture (2 : 1) in a 100 c.c. graduated flask. The contents were allowed to boil for about one minute by immersion in a water-bath, cooled and made up to volume with alcohol-ether. The alcohol-ether extract was then rapidly filtered (care being taken to avoid evaporation during filtration) and an aliquot portion (*e.g. 20 c.c.*) of the filtrate pipetted into a 100 c.c. Pyrex flask and evaporated to dryness. The phosphorus in the residue was then estimated in the same way as for the total phosphorus (*cf.* Kay and Byrom, 1927; Randles and Knudson, 1922).

As these methods of analysis had not been previously applied to bacteriological media, it was deemed advisable to test them to determine whether any of the constituents of the media interfered with the accuracy of the estimations. The inorganic phosphate determinations on ordinary 1% peptone (Parke Davis) bouillon were checked against the magnesium citrate precipitation method. This involved preliminary experiments to determine the delicacy of the magnesium citrate method with phosphate solutions of extreme dilution. A solution of potassium dihydrogen phosphate containing 0·200 mgm. per c.c. was used and treated with an equal volume of magnesium citrate mixture and one-fifth its volume of concentrated ammonia. The precipitate obtained after standing 24 hours was collected and washed, dissolved in 6% trichloracetic acid and diluted to a known volume. The inorganic phosphorus content of an aliquot portion of this solution was then determined in triplicate by Bell and Doisy's method. The values found in this way were 0·191, 0·189 and 0·197 mgm. Evidently, in solutions of this order of concentration, the error is approximately 0·008 mgm., and it is therefore necessary to make this correction with values found by the magnesium citrate method. The phosphate content of the peptone bouillon was next determined by the magnesium citrate method, the details of technique being similar to those just described. The triplicate values for 2 c.c. of bouillon were 0·192, 0·192 and 0·190 mgm., which give (with the correction of 0·008 mgm. previously determined) a final value of 0·200 mgm. Triplicate values obtained by direct application of Bell and Doisy's method to the bouillon were 0·102, 0·106 and 0·106 mgm. per c.c., and the sufficient accuracy of the method is therefore established. A complete analysis of autoclaved solutions of double strength bouillon, sodium β-glycerophosphate and a lecithin emulsion were next undertaken by the colorimetric methods previously described. From the values obtained, theoretical values were deduced for a mixture of known amounts of the three solutions and compared with those found by experiment. The values are as follows:

(*Mgm. per c.c.*)

	Bouillon (double strength).	Glycero-phosphate.	Lecithin.	Mixture.	
				Theor.	Found.
Total P.	0·374	0·370	0·412	0·383	0·376
Inorganic P.	0·243	*Nil*	*Nil*	0·087	0·092
Lipin P.	0·018	0·009	0·390	0·121	0·128

There is a slight "apparent" lipin phosphorus content of the glycerophosphate solution, but this only amounts to 2·5% of the total organic phosphorus present. The foregoing experiments indicate that the methods of analysis of the media described are substantially accurate for the conditions employed. The first medium to be tried was 1% peptone (Parke Davis) bouillon, but the total phosphorus content was only about 0·20 mgm. per c.c., and the changes produced, although indicating some hydrolysis, were so small as to be only just outside the margin of experimental error. Table I shows the results of such an experiment.

TABLE I.—*Phosphorus Distribution (mgm. per c.c.) in Peptone Bouillon after Growth of Certain Organisms.*

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·200	0·154	0·046	0·021
B. coli	0·200	0·160	0·040	0·011
Staphylococcus	0·200	0·160	0·040	0·014
B. dysenteriæ (Shiga)	0·200	0·160	0·040	0·010

Owing to the small amount of total phosphorus present, it was decided to use media to which various organic phosphorus compounds had been added. Lecithin (B.D.H. ovo-lecithin) was first tried on account of its importance as a cell constituent and in view of Stoklasa's findings with synthetic media (1911).

An approximately 1% colloidal solution of lecithin in water was prepared by adding a warm alcoholic solution to boiling water, driving off the alcohol, cooling and making up to the requisite volume. To this was added an equal volume of double strength peptone bouillon, and the medium autoclaved, the pH being adjusted both before and after the autoclaving to 7·4–7·6. Table II shows the results of some experiments made with this medium.

TABLE II.—*Phosphorus Distribution (mgm. per c.c.) after Growth in Peptone Bouillon plus Lecithin.*

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·500	0·390	0·110	0·103
*B. coli**	0·500	0·418	0·082	0·089
Staphylococcus*	0·500	0·394	0·106	0·098
*V. choleræ**	0·500	0·410	0·090	0·075
Control	0·457	0·238	0·219	0·181
B. subtilis	0·457	0·251	0·206	0·172
B. pyocyaneus	0·457	0·241	0·216	0·165
B. diphtheriæ	0·457	0·232	0·225	0·172
B. dysenteriæ (Flexner)	0·457	0·267	0·188	0·177

* After 24 hours' growth only.

Experiments were then carried out with a medium consisting of equal volumes of a solution of sodium β-glycerophosphate of pH 7·2 and double strength peptone bouillon. The strength of the glycerophosphate solution was

such that it contained about 1·0 mgm. phosphorus per c.c. The results given in Table III are typical and seem to indicate, from the relatively large increase in inorganic phosphate, that glycerophosphate is more readily hydrolyzed than lecithin.

TABLE III.—*Medium Containing Peptone Bouillon plus Na β-glycerophosphate (mgm. per c.c.)*

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·714	0·425	0·289	0·023
B. coli	0·714	0·578	0·136	0·008
Staphylococcus	0·714	0·511	0·203	0·013

We were therefore led to investigate the action of different bacteria in peptone bouillon to which were added approximately equal amounts of organic phosphorus as lecithin and glycerophosphate. The lecithin-glycerophosphate emulsion was prepared by adding a warm alcoholic solution of 1 gm. lecithin to 100 c.c. of a boiling solution of glycerophosphate (containing about 0·5 mgm. organic phosphorus per c.c.), boiling off the alcohol and making up to 100 c.c. with distilled water. Table IV gives some results with a number of organisms.

TABLE IV.—*Medium Containing Peptone Bouillon plus Lecithin Na β-glycerophosphate (mgm. per c.c).*

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·765	0·268	0·497	0·216
B. coli	0·765	0·428	0·337	0·190
Staphylococcus	0·765	0·346	0·419	0·20?
B. pyocyaneus	0·765	0·310	0·455	0·158
Control	0·694	0·201	0·493	0·231
B. paratyphosus A	0·694	0·323	0·371	0·198
Streptococcus	0·694	0·256	0·438	0·229
B. proteus	0·694	0·388	0·306	0·193
B. prodigiosus	0·694	0·320	0·374	0·190
Anthracoid bacillus	0·694	0·200	0·494	0·229
Control	0·742	0·268	0·474	0·214
V. cholerœ (Freeman)	0·742	0·366	0·376	0·172
V. cholerœ (2463)	0·472	0·466	0·276	0·133
Control	0·790	0·289	0·501	0·226
B. typhosus	0·790	0·348	0·442	0·221
B. diphtheriæ (1)*	0·790	0·292	0·498	0·231
B. diphtheriæ (2)*	0·790	0·300	0·490	0·235
B. diphtheriæ (Park)*	0·790	0·294	0·496	0·235

* Three different strains.

Various organisms of the typhoid-dysentery group were next tested together in this medium with a view to obtaining comparable results as to their utilization of phosphorus, the results appearing in Table V.

TABLE V.*—(*Mgm. per c.c.*)

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·820	0·336	0·484	0·235
B. paratyphosus A	0·820	0·371	0·449	0·229
B. paratyphosus B	0·820	0·409	0·411	0·203
B. paratyphosus C	0·820	0·360	0·460	0·222
B. enteritidis (Gaertner)	0·820	0·409	0·411	0·194
Control	0·780	0·383	0·397	0·227
B. typhosus	0·780	0·462	0·318	0·200
B. dysenteriæ (Flexner)	0·780	0·424	0·356	0·211
B. dysenteriæ (Shiga)	0·780	0·414	0·366	0·225

* Duplicate inoculations were made in each case (three with Shiga) and the results (not given in the table) agreed very closely with those recorded above.

We may now consider briefly the results obtained up to this point. Most of the organisms of the typhoid-dysentery group utilize lipin phosphorus, though *B. dysenteriæ* (Shiga) appears to be an exception under these conditions, and *B. typhosus* does so to a less extent than other members of the group. An increase in inorganic phosphate is also observed in all cases, the amount varying considerably with different members. With *V. choleræ* considerable utilization of lipin phosphorus occurs, the only other organism to show a similar and even greater utilization being *B. pyocyaneus*, though with the latter there is not such a large increase in inorganic phosphate. *B. proteus* and *B. prodigiosus* both show considerable utilization of lipin phosphorus accompanied by a relatively large increase in inorganic phosphate. With *B. diphtheriæ* and the anthracoid bacillus no significant changes in the phosphorus distribution in the media could be observed with this technique. Staphylococcus and streptococcus produce appreciable amounts of inorganic phosphate from the organic phosphorus compounds, presumably largely at the expense of the glycerophosphate, as the lipin phosphorus content of the medium is only reduced to a very slight extent.

At this stage it was decided to modify the medium in order that some of the more delicate organisms might be used, including at the same time some of those organisms which had been previously grown in the ordinary 1% peptone bouillon *plus* lecithin and glycerophosphate. Sufficient sterile rabbit serum, heated for one hour at 60°C., was added to the lecithin-glycerophosphate bouillon to bring the concentration of serum in the mixture up to 20%. 48-hour cultures were used as before. Some of the results obtained are given in Table VI.

TABLE VI.—*Lecithin-glycerophosphate Peptone Bouillon plus Rabbit Serum (mgm. per c.c.)*

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·526	0·145	0·381	0·156
B. pyocyaneus	0·526	0·187	0·339	0·119
Staphylococcus albus	0·526	0·154	0·372	0·156
B. paratyphosus B	0·526	0·282	0·244	0·137
B. proteus	0·526	0·307	0·219	0·144
Meningococcus (1)	0·526	0·151	0·375	0·160
Meningococcus (2)	0·526	0·148	0·378	0·160
Control	0·552	0·172	0·380	0·157
B. subtilis	0·552	0·172	0·380	0·152
Streptococcus hæmolyticus	0·552	0·191	0·361	0·159
V. cholerœ	0·552	0·230	0·322	0·133
Control	0·497	0·155	0·342	0·152
B. melitensis	0·497	0·155	0·342	0·154
Micrococcus catarrhalis (6)	0·497	0·155	0·342	0·150
Micrococcus catarrhalis (M.)	0·497	0·155	0·342	0·147

It is interesting to note that no change could be detected in the media inoculated with Meningococcus, although it should be noted that growth was not so vigorous as with the other organisms. A further experiment, in which a heavy inoculation was made, confirmed these findings. *Staphylococcus albus*, *B. melitensis* and *Micrococcus catarrhalis* also showed no appreciable hydrolysis. The other organisms gave results which agreed well with those obtained in the medium without serum.

As was previously mentioned, all our experiments up to this point had been carried out on whole cultures, and it occurred to us that some valuable information with regard to the fate of lipin phosphorus in cultures of *B. pyocyaneus* might be obtained by using both whole and centrifuged cultures in the same experiment. In three separate experiments (using ordinary peptone bouillon with lecithin and glycerophosphate added) in which the cultures were centrifuged at high speed for 1½–2 hours, the inorganic phosphate, the organic acid-soluble phosphorus and the lipin phosphorus were found to be almost identical with the values obtained for the whole cultures, and, in two of these, the phosphorus partition of the bacteria themselves was examined using the following technique: 60 c.c. of the whole culture were centrifuged for 2 hours, and the supernatant fluid decanted, the residual bacteria being washed with about 60 c.c. of 0·85% saline, centrifuged again and the saline decanted. The bacteria were then suspended in saline and the volume made up to 10 c.c. The total phosphorus content was then determined on 2 c.c. of the suspension. With *B. pyocyaneus* the value obtained was 0·817 mgm. The lipin phosphorus in 5 c.c. of the suspension in the same experiment was found to be 0·174 mgm. This value, expressed as a percentage of the total phosphorus of the bacteria, is approximately 7%, so

that lipin phosphorus only accounts, at most, for a small proportion of the phosphorus of the bacteria which is thus shown to be in some state of organic combination other than lipin. Its amount (approximately 0·064 mgm. per c.c.) is almost equal to the lipin phosphorus hydrolyzed per c.c. of medium (0·072 mgm.) (*cf.* Table VII).

TABLE VII.—(*Mgm. per c.c.*)

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·805	0·267	0·538	0·225
B. pyocyaneus	0·805	0·301	0·504	0·153

These facts lend support to the view that the lipin phosphorus of the medium which is utilized is first hydrolyzed to inorganic phosphate and then re-synthesized into some other organic phosphorus compound, which is an essential part of the bacterial protoplasm. A further experiment was carried out with a three days' culture of *B. pyocyaneus*. Very good growth was obtained, and more than 50% of the total lipin phosphorus of the medium was hydrolyzed (lipin phosphorus content of the control medium = 0·213, and of the culture, 0·091 mgm. per c.c.) The results confirm the findings of the previous experiment.

A similar experiment with a four days' culture of *B. coli* gave the following results:

TABLE VIII.—(*Mgm. per c.c.*)

	Total P.	Inorg. P.	Total org. P.	Lipin P.
Control	0·920	0·372	0·548	0·200
B. coli	0·920	0·572	0·348	0·182

The large increase of inorganic phosphate would indicate that the process of hydrolysis of the organic phosphorus compounds of the medium takes place considerably more rapidly than the process of re-synthesis to the organic phosphorus compound of the bacteria, thus causing an accumulation of inorganic phosphate in the medium.

DISCUSSION.

From a consideration of the results obtained with some two dozen organisms, detailed in the foregoing tables, there emerge several interesting facts. It would appear that organisms are able to utilize organic phosphorus in different ways, under the conditions and with the media used in our experiments. A large group of organisms has been found to utilize both lecithin and glycerophosphate. In this category are Streptococcus (both hæmolytic and non-hæmolytic), *B. dysenteriæ* (Flexner), *B. proteus vulgaris*, *B. typhosus*, *B. paratyphosus* A, B and C, *B. prodigiosus*, *V. choleræ*, and *Staphylococcus aureus*. Of these, *V. choleræ*, *B. paratyphosus* B and C, and *B. dysenteriæ* (Flexner) utilize more lipin phosphorus than other members of the group in 48-hour old cultures. The apparent more ready decomposition of glycerophosphate than lecithin by many organisms may be due to the fact

that in the latter there are two linkages to be hydrolyzed before inorganic phosphate can be liberated, compared with only one in glycerophosphate. *B. pyocyaneus* would appear to show a preference for lipin phosphorus over other forms of organic phosphorus such as glycerophosphate. A study of the phosphorus partition in *B. pyocyaneus* by centrifugation of the culture shows that the sum of the phosphorus of the bacteria and the increase in inorganic phosphorus (as orthophosphoric acid) is approximately equal to the amount of lipin phosphorus utilized. Evidence has also been presented which suggests that the mechanism of incorporation of phosphorus in the bacterial protoplasm depends on the initial hydrolysis of organic phosphorus compounds (whether lecithin or glycerophosphate) followed by re-synthesis into the particular compound required by the organism. Many previous workers, notably Stoklasa, have supposed this compound to be a nucleoprotein.

It has not been possible to demonstrate the mechanism of phosphorus utilization by some of the more delicately growing organisms such as Meningococcus, *Micrococcus catarrhalis* or Gonococcus by the technique employed in the present work.

SUMMARY.

(1) A method has been elaborated for determining the phosphorus distribution in bacterial cultures, and its applicability to bacterial media established.

(2) In the case of *B. coli* the lipin phosphorus forms only a minor part of the total organic phosphorus hydrolyzed during growth, and in this respect the bulk of the organisms investigated resemble *B. coli*, the actual proportions of lipin and non-lipin phosphorus hydrolyzed varying with the individual organism.

(3) *B. pyocyaneus* appears to utilize mainly lipin phosphorus.

(4) In some few cases the composition of the bacteria themselves has been investigated with regard to phosphorus distribution.

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ON THE EFFECT OF ULTRA-VIOLET IRRADIATION UPON THE RESISTANCE OF MICE EXPOSED TO *PASTEURELLA* INFECTION.

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THE object of the experiment described in this paper was to test upon a numerically adequate sample of animals exposed to infection under approximately normal conditions whether irradiation conferred any immunity from fatal infection.

The published results with regard to the resistance of irradiated animals to direct experimental infection, not infrequently based upon scanty data, have been indecisive, although, perhaps, inclining towards an unfavourable conclusion. For instance, Mayer (1924) reached indecisive results in respect of guinea pigs infected with tubercle bacilli by inhalation; C. M. Hill and Clarke (1927) also reached negative results using rats inoculated with pneumococcus. These rats were shaved before irradiation and in some burns were produced. L. Hill, Colebrook and Eidenow (1924) found that a dose of ultra-violet rays just sufficient to produce an erythema applied to an area of skin equivalent to 20–30 cm.^{2} per kilo of body weight increased the hæmobactericidal power of the blood against staphylococci, when the blood was collected two hours after irradiation and tested *in vitro* by the "slide cell" technique of Wright, Colebrook and Storer (1923). Eidenow (1929) recorded results from which he concluded that the bactericidal substance was produced locally, at the site of irradiation, whence it was carried into the blood stream; so far, however, his experiments have failed to demonstrate any bactericidal activity in extracts of irradiated tissues. Indeed, irradiated blood seems to have less than normal bactericidal power although the injection of such blood *in vivo* appears to increase the bactericidal action of the recipient's blood when tested *in vitro* against staphylococci. There is evidence that irradiation of the skin increases the number of blood platelets and may increase the number of polymorphonuclear cells and lymphocytes. According to Eidenow, 48 hours' irradiation of shaved rabbits with a lamp emitting infra-red luminous and ultra-violet rays as far as 2850 Å.U., but no shorter rays, and giving a light corresponding fairly well to sunlight, increased the resistance of the animals to a dose of staphylococci intravenously injected immediately after the light bath, a dose which was lethal to control rabbits in 24 hours.

Such irradiation did not produce noticeable erythema of the skin, but increased the hæmo-bactericidal power and induced a leucocytosis. Irradiation after infection gave no noteworthy protection. The effects of irradiation seem to be transient; by it the hæmo-bactericidal power cannot be maintained at a higher level for a prolonged period. The increased resistance to staphylococcal infection possessed by the irradiated rabbits was shown by the fact that while all were made unwell by the infection, some made a progressive recovery, while the remainder lost weight and died, but only after from 3 to 11 days of pyæmia.

Similar experiments carried out by L. Hill and Eidenow (1930) using gas fires fitted with "beam radiants" as fuel gave similar results. This fuel gives off short infra-red rays which, together with red rays, have power to penetrate the epidermis. The fires warmed up the bodies of the shaved rabbits two or three degrees Fahrenheit; but, as a source of dark heat giving a like warming effect did not increase resistance to staphylococcic infection, this resistance cannot be attributed to raised body temperature.

It seemed therefore worth while to determine whether a prolonged light bath would afford any protection to mice exposed to a respiratory infection. Against the likelihood of such advantage being conferred was the fact that, under the conditions of natural infection in a mouse community, the light bath must take place one or two, even perhaps several, days before infection and, as we have seen, there is no experimental evidence that protection afforded by light baths will persist so long. Again light treatment which clinical experience has shown to be most effective for lupus, infected wounds, carbuncles, boils, impetigo, etc., is in this case acting on infections localized in the skin. It has, however, been claimed that irradiation once a week during the winter diminished the number of "colds" among students at a university selected on account of their susceptibility to "colds."

On 24 . 4 . 29 an infected herd was formed by inoculating 25 mice with *Pasteurella* and adding them to 100 normal mice, housed in the type of cage employed in the study of experimental epidemics. Thereafter 3 normal mice were added to the cage each day. By 10 . 5 . 29 all the inoculated mice were dead, and no effective spread to the contact mice had yet occurred. On that day a second batch of 25 inoculated mice were added to the cage. Within the next few days some of the contacts died of typical pasteurellosis, and on 15 . 5 . 29 the experiment began by the daily addition of 3 irradiated mice at the same time as the 3 regular controls. The treatment of the irradiated mice was as follows. The lamp employed consisted of a frosted vitrosil tubular mercury vapour lamp, screened by a sheet of glass permeable to ultra-violet rays up to 2850 Å.U. but not to shorter rays, along which on either side was placed a row of incandescent lamps sufficient in number to balance the blue light coming from the mercury vapour lamp and produce colours, *e. g.* of flowers, equivalent to those produced by sunlight. The animals were placed in cages four feet below the lamp and exposed to the light for 48 hours. A fan was kept running to keep the cages cool enough. The cages were at first covered with gauze which allowed about half the light to penetrate, later this was done away with and finally (viz. from 17 . 7 . 29) the screen of glass was removed from the lamp to allow shorter ultra-violet

rays to pass. As will appear, the change did not alter the negative character of the results. It should be noted that the mice were not shaved and thus differed from the rabbits tested with staphylococci. It has, however, been shown that exposure of the naked parts of the head and feet to irradiation suffices to protect young rats fed on a ricket-producing diet against rickets, and it would therefore seem probable that the mice used in this experiment received an effective exposure, so far as the anti-rachitic effect can be accepted as a criterion of adequate dosage.

On 22 . 8 . 29 six normal controls and no irradiated mice were added but, with this exception, there was no departure from the rule of adding daily three of each class. On 30 . 10 . 29 the last batch of immigrants was added and on 31 . 12 . 29 the surviving members of the colony were killed.

Throughout the experiment all dead mice which were not eaten by their companions were submitted to the usual post-mortem examination, including the preparation of cultures from the heart's blood and from the spleen.

Table I sets out the general vital statistics of the community* from which it appears that the course of events differed in no significant way from that usually observed in such colonies.

The planning of the experiment admitted of a direct comparison of the fates of normal and irradiated mice. Thus of the entrants to the community down to 16.7.29 there were 189 irradiated and 189 normal mice admitted on the same days and all of these died before the experiment was brought to an end (see Table II). The mortality of the irradiated mice from the specific infection was insignificantly less and from other causes insignificantly greater than that of the control mice. The average life time of the controls was rather greater, when all causes of mortality were considered, than that of the irradiated mice. When mortality from all causes save the specific infection was ignored the two expectations of life were identical.

For the period during which the irradiation dosage was greater, the number of controls (321) exceeded by 6 the treated animals (315) and at the end of the experiment one animal of each group survived. Again a non-significant difference in favour of the irradiated mice is present in the casualty list from specific infection, a slightly heavier adverse balance against the irradiated animals under the heading of other causes of death. Summarizing the results by means of the expectation of life at entrance to the community, we find, as in the first part of the experiment, that the controls lived rather longer than the irradiated mice when all causes of death are brought into the account and quite as long when only the specific cause of mortality is considered. It is quite clear that irradiation conferred no advantage upon the treated animals in respect of the hazards to which they were exposed. Considering the scale of the experiment, which has involved the use of more than 1000 mice, it is improbable that the vagaries of sampling have concealed any really measurable advantage.

* To save space, this table is abbreviated, values being given (after the first 10 days) at intervals of 10 days.

TABLE I.—*Life Table for Irradiated Mice* (15.5.29–16.7.29 *inclusive*).*

SPECIFIC DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	29·00
1	10000	..	..	28·00
2	10000	..	..	27·00
3	10000	..	..	26·00
4	10000	..	..	25·00
5	10000	..	..	24·00
6	10000	52·91	·00529	23·00
7	9947·09	158·73	·01596	22·12
8	9788·36	105·82	·01081	21·47
9	9682·54	52·91	·00546	20·70
10	9629·63	53·20	·00552	19·81
20	6671·80	632·67	·09483	15·75
30	2395·56	378·25	·15789	27·14
40	1040·51	..	..	47·71
50	864·42	..	..	46·79
60	370·47	..	..	91·50

TOTAL DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	24·53
1	10000	..	..	23·53
2	10000	..	..	22·53
3	10000	..	..	21·53
4	10000	..	..	20·53
5	10000	..	..	19·53
6	10000	52·91	·00529	18·53
7	9947·09	158·73	·01596	17·62
8	9788·36	105·82	·01081	16·90
9	9682·54	105·82	·01093	16·08
10	9576·72	52·91	·00552	15·25
20	6136·56	582·01	·09483	10·42
30	2010·58	317·46	·15789	13·94
40	740·74	52·91	·07143	22·06
50	370·37	..	..	30·47
60	158·73	..	..	53·44

* The column headed $\mathring{e}_x$ gives the average after-lifetime, in days, of mice who survived to the beginning of day x.

TABLE I (continued).—*Life Table for Irradiated Mice* (17.7.29–30.10.29 *inclusive*).

SPECIFIC DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	26·33
1	10000	31·85	·00318	25·33
2	9968·15	..	..	24·41
3	9968·15	..	..	23·41
4	9968·15	31·95	·00321	22·41
5	9936·20	95·85	·00965	21·48
6	9840·36	95·85	·00974	20·69
7	9744·51	159·75	·00164	19·89
8	9584·76	127·80	·01333	19·21
9	9456·97	127·80	·01351	18·46
10	9329·17	159·75	·01712	17·71
20	5254·93	682·46	·12987	16·16
30	2142·72	76·53	·03571	24·45
40	1530·35	162·04	·12000	26·01
50	815·03	81·50	·10000	30·74
60	570·52	..	..	32·99

TOTAL DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	31·75	·00317	23·08
1	9968·25	31·75	·00318	22·15
2	9936·51	..	..	21·22
3	9936·51	31·75	·00319	20·22
4	9904·76	31·75	·00321	19·29
5	9873·02	95·24	·00965	18·35
6	9777·78	95·24	·00974	17·52
7	9682·54	158·73	·01639	16·69
8	9523·81	126·98	·01333	15·96
9	9396·83	126·98	·01351	15·17
10	9269·84	158·73	·01712	14·37
20	4888·89	634·92	·12987	11·36
30	1777·78	95·24	·05357	14·86
40	793·65	126·98	·16000	17·58
50	317·46	31·75	·10000	28·10
60	222·22	..	..	29·22

TABLE I (continued).—*Life Table for Controls* (15.5.29–16.7.29 *inclusive*).

SPECIFIC DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	29·17
1	10000	..	..	28·17
2	10000	..	..	27·17
3	10000	..	..	26·17
4	10000	..	..	25·17
5	10000	53·48	·00535	24·17
6	9946·52	..	..	23·30
7	9946·52	53·76	·00541	22·30
8	9892·76	53·77	·00543	21·42
9	9838·99	53·77	·00546	20·53
10	9785·23	54·06	·00552	19·64
20	6271·60	287·69	·04587	17·06
30	2783·62	63·26	·02273	23·16
40	1447·93	..	..	29·49
50	1158·35	144·79	·12500	25·99
60	756·03	..	..	27·12

TOTAL DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	52·91	·00529	26·06
1	9947·09	..	..	25·20
2	9947·09	52·91	·00532	24·20
3	9894·18	..	..	23·32
4	9884·18	..	..	22·32
5	9884·18	105·82	·01070	21·32
6	9788·36	..	..	20·55
7	9788·36	52·91	·00541	19·55
8	9735·45	52·91	·00543	18·65
9	9682·54	105·82	·01093	17·75
10	9576·72	105·82	·01105	16·94
20	5767·20	317·46	·05505	14·01
30	2328·04	105·82	·04545	18·48
40	1058·20	..	..	24·25
50	846·56	158·73	·18750	19·44
60	423·28	..	..	24·00

TABLE I (continued).—*Life Table for Controls* (17.7.29–30.10.29 *inclusive*).

SPECIFIC DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	26·90
1	10000	..	..	25·90
2	10000	..	..	24·90
3	10000	..	..	23·90
4	10000	..	..	22·90
5	10000	62·31	·00623	21·90
6	9937·70	31·15	·00313	21·04
7	9906·54	62·31	·00629	20·10
8	9844·24	62·50	·00635	19·23
9	9781·73	62·50	·00639	18·35
10	9719·23	31·25	·00322	17·46
20	6579·82	529·03	·08040	12·49
30	2556·49	37·05	·01450	15·73
40	1239·58	177·08	·14286	16·52
50	531·25	..	..	24·14
60	354·17	..	..	24·08

TOTAL DEATHS.

Cage age in days.	l_x.	d_x.	q_x.	$\mathring{e}_x$.
0	10000	..	..	25·07
1	10000	..	..	24·07
2	10000	..	..	23·07
3	10000	..	..	22·07
4	10000	..	..	21·07
5	10000	62·31	·00623	20·07
6	9937·70	31·15	·00313	19·19
7	9906·54	93·46	·00943	18·21
8	9813·08	62·31	·00635	17·42
9	9750·78	62·31	·00639	16·53
10	9688·47	31·15	·00322	15·63
20	6199·38	498·44	·08040	10·66
30	2149·53	31·15	·01449	12·85
40	872·27	124·61	·14286	13·75
50	373·83	..	..	17·67
60	218·07	31·15	·14286	16·50

TABLE I (continued).—*Probability of Dying in Next 5 Days.*

Cage age in days.	Specific deaths. 15.5.29–16.7.29. Controls.	Irradiated.	Specific deaths. 17.7.29–30.10.29. Controls.	Irradiated.	Total deaths. 15.5.29–16.7.29. Controls.	Irradiated.	Total deaths. 17.7.29–30.10.29. Controls.	Irradiated.
0	·0000	·0000	·0000	·0064	·0106	·0000	·0000	·0127
1	·0053	·0000	·0062	·0160	·0160	·0000	·0062	·0191
2	·0053	·0053	·0093	·0224	·0160	·0053	·0094	·0256
3	·0107	·0212	·0156	·0385	·0160	·0212	·0187	·0415
4	·0161	·0317	·0218	·0513	·0214	·0317	·0250	·0513
5	·0215	·0370	·0281	·0611	·0321	·0423	·0312	·0611
6	·0217	·0424	·0251	·0682	·0324	·0476	·0282	·0682
7	·0326	·0533	·0252	·0656	·0432	·0691	·0314	·0689
8	·0383	·0435	·0382	·0667	·0543	·0703	·0444	·0733
9	·0497	·0499	·0513	·0847	·0765	·0874	·0607	·0946
10	·0897	·0732	·0614	·0930	·1105	·1160	·0804	·1164
20	·3701	·4336	·4012	·4124	·3853	·4483	·4171	·4351
30	·2089	·4105	·2065	·2480	·2273	·4737	·2609	·3571
40	·1500	·1692	·3929	·2527	·1500	·3571	·3929	·3600
50	·1250	·1429	·2500	·3000	·1875	·1429	·2500	·3000
60	·2500	·0000	·3333	·0000	·2500	·0000	·4286	·0000

TABLE II.

	Period 15.5.29–16.7.29. Irradiated mice.	Normals.	Period 17.7.29–30.10.29. Irradiated mice.	Normals.
Number of mice concerned	189	189	315	321
„ of mouse-days exposed to risk	4728	5020	7425	8207
Number of deaths :				
Pasteurella	126	132	213	229
Not examined*	33	30	54	53
Nil found	30	27	47	38
Number of survivors	..	..	1	1
Expectation of life at age 0 :				
Specific	29·00	29·17	26·33	26·90
Total	24·53	26·06	23·08	25·07

* *I.e.* mice devoured, or partly devoured, by their comrades, the remains being unsuitable for examination

We desire to thank the colleagues in our respective departments who have collaborated in this investigation, particularly Dr. Eidenow, Mrs. Wilson and Mr. Martin.

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THE ELECTRICAL CHARGE OF THE VIRUS OF FOWL-POX AND THE AGENT OF THE ROUS SARCOMA.

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OF the methods available for determining the electrical charges carried by virus particles, that described by Bedson and Bland (1929) has certain advantages in that it is extremely simple, requires no expensive apparatus and appears to give reliable information. The "blotting-paper" method has been here applied in an investigation of the electrical charges carried by the virus of fowl-pox and by the agent of the Rous sarcoma No. 1. The technique of the experiments was similar to that employed by Bedson and Bland.

EXPERIMENTS WITH FOWL-POX VIRUS.

Two strains of fowl-pox virus were employed, the results being similar in both cases. An actively growing lesion was removed from the breast of a pigeon and ground up in the appropriate buffer solution to make a dilution of 1 in 10. The suspension was then lightly centrifuged to throw down gross particles, the supernatant liquid pipetted off and placed in sterile watch-glasses in which strips of blotting-paper were suspended to a depth of 0·5 cm. The fluid was allowed to ascend the strips to a height of 10 cm., after which centimetre lengths 3 to 7 inclusive were removed and ground up separately in a little of the same buffer solution. The suspension was then applied to the skin of the breast of pigeons from which the feathers had been previously removed.

Virus was found to be present in the centimetre lengths 3 to 7 at pH 6·6, 7·0, 7·2, 7·6 and 8·0.

It is therefore concluded that over this range of pH the virus of fowl-pox has, relatively to the blotting-paper, a negative charge.

EXPERIMENTS WITH THE ROUS SARCOMA.

Similar experiments were carried out with strains of the Rous sarcoma No. 1, for which I am indebted to Prof. Leitch and to Prof. McIntosh.

Actively growing tumours were aseptically removed and ground up with sterile sand in the appropriate buffer solutions. The suspensions were then centrifuged and the supernatant fluid placed in watch-glasses in which strips of filter-paper were suspended to a depth of 0·5 cm. The experiments were carried out at pH 5·6, 6·6, 7·2 and 8·0.

In the experiments with the fowl-pox virus the fluid rapidly ascended to the full height of the filter-paper, but in these experiments the fluid never passed up for more than 5·5 cm., the average being about 4·0 cm. Centimetre lengths 3 and upwards, into which the fluid had passed, were removed, ground up in a mortar with the appropriate buffer solution, and together with a little sterile kieselguhr injected into the pectoral muscles of chickens. When single centimetre lengths were inoculated, no evidence was obtained that the agent of the Rous sarcoma ascends the filter-paper. When, however, centimetre lengths 3 and upwards were inoculated together into the same chick, positive results were obtained. It is therefore concluded that over the range of pH 5·6 to 8·0 the agent of the Rous sarcoma is, relatively to the blotting-paper, negatively charged. This question is being further investigated using a cataphoresis apparatus.

CONCLUSIONS.

The virus of fowl-pox is negatively charged at pH 6·6 to 8·0. The agent of the Rous sarcoma is negatively charged at pH 5·6 to 8·0.

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SOME PROPERTIES OF MUCUS, WITH SPECIAL REFERENCE TO ITS ANTIBACTERIAL FUNCTIONS.

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FLEMING (1922 and 1929) and Fleming and Allison (1922) have described an enzyme, lysozyme, which has remarkable antibacterial properties. They find that this is widely spread in animal tissues and occurs even in vegetables. Its antibacterial qualities are exhibited in three ways: (1) Inhibition of growth; (2) killing without lysis; (3) killing accompanied by lysis. Of the substances so far examined, human tears and hen-egg white are richest in lysozyme. Fleming is inclined to connect it with some form of natural immunity.

The alimentary canal is a potential portal of entry for bacteria of many kinds. The contents of its lumen may provide a suitable pabulum for growth preliminary to actual penetration of the mucosa. It therefore becomes of some interest to consider whether lysozyme enters into a normal protective mechanism resident in the mucosa and its secretions.

The experiments herein described have as their object the elucidation of this problem.

(A) ANTIBACTERIAL PROPERTIES OF MUCUS.

Cats mainly have been used, but comparative experiments have been done on the dog, rabbit, guinea-pig and goat.

While the broader question of a possible protective complex of factors operating in the alimentary canal has been kept in mind, our investigations have been mainly concerned with the mucosa and its secreted mucus.

Collection of Mucus.

The mucus has, in general, been collected in the following ways:—(*a*) Saliva: A flow of this was caused by intravenous injection of pilocarpine into either

* With a grant from the Medical Research Council.

anæsthetized or decerebrate animals. The saliva was collected as it dropped from the mouth. (*b*) Colon mucus: For the majority of experiments colonic mucus has been used, as this can be readily obtained free from enzymes present in other parts of the alimentary canal. The abdomen was opened by a longitudinal incision, and the colon was pulled out. A loop of string was tied just distal to the ileo-cæcal junction, and another loop just proximal to the point where the inferior mesenteric vessels apply themselves to the surface of the rectum (in the case of the cat and dog). With a cautery the gut was opened just distal to the first and proximal to the second ligature. In the case of the herbivores, distal segments of the colon were used. The fæces were then thoroughly cleaned out by irrigation with warm normal saline. When completely clean a ligature was tied to occlude the lower opening, and a large glass cannula tied into the upper opening. (1) In some experiments a reasonably clear solution of mucus in saline was desired. To secure this, warm saline was injected through the cannula, and the fluid moved over the mucosa by squeezing the gut. After a few minutes of this treatment the fluid was tipped out into a tube and centrifugalized to remove gross *débris*. The resulting fluid will throughout this paper be referred to as colon washings. (2) In other experiments a weak solution of mustard oil in olive oil was inserted through the cannula, which was then corked. The oil was left about two hours, and then the whole contents of the lumen were evacuated into a clean dish. The mucus was sucked off by a pipette and centrifugalized to remove excess of oil. The mucus secretion thus secured was generally very viscid, and contained whitish inclusions embedded in a thinner clear matrix. It was not found possible to separate these two portions satisfactorily either by centrifugalization, pipetting or filtration through a thin layer of glass-wool.

During the progress of the work it was decided to extend the observations to other secretions, namely, tears and nasal secretion. These again have generally been secured by the action of pilocarpine. In order to secure reflexly-produced tears for comparison with pilocarpine-produced tears, the cornea and conjunctiva of decerebrate cats were stimulated faradically and the tears collected as they formed with a pipette.

Test Organisms.

Fleming (1922) states that 75% of air organisms are sensitive to the action of lysozyme. Most of his work was done on *M. lysodeikticus*, and we are indebted to him for supplying us with a culture of this micrococcus, which will be referred to in this communication as organism No. 13.

To obtain other suitable organisms ordinary agar plates were exposed to the air of the laboratory for about an hour, then incubated at 37° C. for 24 hours, after which the plates remained at room temperature for a further 24 to 48 hours. About a dozen different colonies were picked off and sub-cultured on agar slants. Emulsions of these organisms were next subjected to the action of fresh hen-egg white acting in a series of dilutions from 1 in 10 to 1 in 5120. It was thus possible to select two specially sensitive organisms, hereinafter known as No. 1 and No. 11, and one moderately sensitive organism (known as No. 3), and one completely resistant to lysis (known as No. 2).

Antibacterial Action.

Various methods were used for the detection of the different degrees of antibacterial activity:

(1) *Inhibitory action.*—The method used is practically identical with that employed by Fleming (1922), and consists essentially in embedding various tissues and secretions beneath a layer of agar, and then sowing the test organism on the surface. In the case of secretions two methods were used: (1) A preliminary dilution with warm melted agar was made, and then a

FIG. 1.—A result obtained with a "well" plate, *M. lysodeikticus* inhibited by cat's saliva. The hole bored in the bottom of the Petri dish is here seen through a layer of agar. (Photo by H. P. Hudson.)

"button" poured into the centre of a Petri dish and allowed to set. This was then completely covered by a thin layer of agar, and the surface of this sown. Plates were read after about 24 hours' incubation. Controls were set up in an identical manner by emulsifying dilute mustard oil in agar, pouring a "button," and then covering it and sowing. No inhibition was noted. (2) The "well" plate method: 1·6 cm. holes were drilled through the centre of Petri dishes 9 cm. in diameter. Each drilled half was sterilized inside a much larger Petri dish. Rubber bungs were chosen so as to fit accurately the holes without protruding into the cavity of the dish. These bungs, after sterilization, were fitted into the sterile dishes. The internal surface of the bung was coated with sterile melted paraffin. This latter procedure was found to be necessary, as

rubber itself gives up some antibacterial substance to the agar. This diffusion into the agar was prevented by the paraffin coating. Melted agar at 45° C. was then poured into the dishes and allowed to set. The test organism was heavily sown broadcast, and the bung withdrawn. The dish was inverted, and the cavity left by the withdrawal of the bung filled completely with the material to be examined. The "well" was closed by a coverslip sealed down with paraffin. After 24 hours' incubation with the "well" below to prevent sepage between the plate and the agar, the plates were examined for inhibition of growth over the area of the "well." Controls, using dilute mustard oil, were set up. No inhibition occurred with this (*vide* Fig. 1).

(2) *Bactericidal action.*—It is obvious that a substance may be capable of exerting a more intense antibacterial action than that of a mere inhibitor; it may actually kill the organisms. Two experiments were planned to test this property in various animal substances. To 0·5 c.c. of the material to be tested there was added 0·1 c.c. of an emulsion (50 million per c.c.) of an organism; after thorough mixing, a sample was plated out and the colonies counted after 24 hours' incubation. The mixture was incubated, and after various intervals a sample was plated out, as before, and the colonies counted. A comparison of the numbers of colonies gives an indication of the bactericidal power of the substances tested.

(3) *Lysis.*—The test organism was grown on agar slopes for 24 hours, washed off in normal saline, and emulsified by shaking for about 1 hour on a mechanical shaker. Using the same standard throughout the whole series of experiments the emulsion, which was prepared fresh each day, was standardized by the opacity method to contain roughly 2000 million organisms per c.c., and the bacteria were *not** subjected to heat before use. The material to be tested was put out in a series of dilutions, $1x$, $1/2x$, $1/4x$, $1/8x$, etc. Controls were set up for the emulsion and for the material under investigation. The various dilutions were first made, in a volume of 0·5 c.c., and then 0·5 c.c. of the test emulsion was added to each tube except the secretion control. The tubes were well shaken and placed in the incubator at 37° to 40° C. for 1 hour, after which a reading was made. A final reading was made next day, after the tubes had stood overnight at room temperature (about 15° C.). A lamp designed by E. G. D. Murray specially for the observation of opacities and particles was used to read the results, which, unless otherwise stated, refer to organism No. 1.

* During a series of experiments (not included in this paper) along lines similar to those described by Allison (1924), an emulsion of *M. lysodeikticus* was prepared in bulk, and used throughout the series, in order to secure uniform conditions as to the dose of the test organism. Fleming (1922) states that lysozyme acts equally well on living and heat-killed bacteria. The stock emulsion was accordingly heated at 60° C. for 30 minutes on each of three successive days, and then tested for sterility. As this state had not been attained, the emulsion (standardized to contain 2000 million per c.c.) was heated for 1 hour at 70° C., after which it was sterile. The results of some experiments upon rabbit's saliva, which was being tested against a living organism (No. 1) and the heat-killed *M. lysodeikticus* (No. 13), led us to suspect that the latter organism was much less susceptible to lysozyme after it had been subjected to heating at 70° C. than before heating. To investigate this point, fresh hen-egg white was tested against *M. lysodeikticus* in two parallel experiments, in one case using the fresh living micrococci, and in the other case the heated emulsion. It was found that the latter's susceptibility to lysozyme was considerably reduced, though not abolished.

Results.

(1) *Inhibitory action:*

(*a*) *Cat secretions and tissues.*—These were tested either by embedding pieces of untreated tissue beneath agar, or by grinding the tissue with sand and saline and then introducing it into "well" plates, or by embedding the dried material in agar plates. The results obtained by these methods have been somewhat discordant with the exception of those relating to mucus (see Table I). In one experiment cat's tears were embedded in agar, but no inhibition resulted.

TABLE I.

Test organisms.	Mucosa of colon.			Mucosa of small intestine.			Colon.	Mucus.
	+	±	−	+	±	−	+	−
No. 1	3	3	4	2	0	1	0	5
„ 2	1	1	3	...	...	...	0	5
„ 11	1	0	4	...	...	...	0	6
„ 13	2	3	4	2	0	0	0	4

In this and subsequent tables + = complete inhibition, ± = considerable inhibition, − = no inhibition. The figures refer to the number of experiments falling into each group in which materials from separate animals were used.

(*b*) *Dog secretions and tissues.*—Mucus from the colon alone was used in these experiments. The technique used is the same as that just described for cat materials. The results are summarized in Table II.

TABLE II.

Test organism.	Inhibition.		
	+	±	−
No. 1	1	0	2
„ 2	0	0	2
„ 11	0	2	1
„ 13	2	1	0

(*c*) *Rabbit materials.*—Mucus from the colon only was used. The results are shown in Table III.

TABLE III.

Test organism.	Inhibition.		
	+	±	−
No. 1	0	0	3
„ 11	0	1	1
„ 13	2	0	1

The following criticisms may be levelled at these results: (*a*) The inadequacy of the cultural methods in so far as some essential constituent of the nutrient agar may be effectively removed by the contents of the "well" by a process of diffusion. To meet this criticism controls were set up. In one case 2% gelatin was placed in the "well" and the usual test organisms sown on the plates. In another case distilled water was used in the "well." In both cases there was no evidence of inhibition of growth. (*b*) A failure on the part of inhibitory substances of the nature of lysozyme to penetrate to the surface of the agar. To anticipate a little, it may be mentioned that cat's saliva contains lysozyme. A "well" plate (see Fig. 1) was therefore prepared with cat's saliva, and the area of resulting complete inhibition is very clearly shown. This demonstrates that the inhibitory substance can diffuse through a layer of agar 1 to 2 mm. thick in effective concentration.

Fleming has shown that lysozyme is insoluble in acetone and is precipitated along with proteins from solutions or tissues. After precipitation it is still capable of diffusing into agar and producing inhibition. Table IV shows the results obtained with acetone dried material.

TABLE IV.

Animal.	Tissue.	Organism. No. 1. +	No. 1. −	No. 11. +	No. 11. −	No. 13. +	No. 13. −
Cat	Stomach	1*	2	1*	2	0	3
	Colon	0	3	0	4	0	4
Dog	Stomach	2	0	2	0	2	0
	Colon	3	0	3	0	3	0
Rabbit	Stomach	0	3	0	3	0	3
	Small intestine	2	1	1	1	3	0
	Colon	5	0	5	0	5	0
Guinea-pig	Stomach	1	2	3	0	3	0
	Small intestine	2	1	1	2	1	2
	Colon	0	3	0	3	0	3

* These experiments showed only partial inhibition.

The results of this series of experiments show that the cat tissues examined appear to possess little, if any, inhibitory properties. The dog, on the other hand, gave uniformly positive results. The rabbit and the guinea-pig obviously possess inhibitory substances which are present in different concentrations in the different organs.

(2) *Bactericidal Action.*

It was found that cat's tears and cat's colon mucus were very slightly if at all lethal to organism No. 13, and not at all to *B. prodigiosus*. Human tears were rapidly and easily able to kill organism No. 13, but egg-white failed entirely to kill *B. prodigiosus*—indeed, it even appeared definitely to act as a medium of growth for this organism.

(3) *Lytic Action.*

Cat secretions.—Using the technique already described, the following typical results were obtained and are set out in Table V.

In five experiments cat tears gave completely negative results against organisms No. 1 and No. 13. In one experiment organism No. 2 was used, and likewise gave negative results. Findlay (1925) states that the tears of the

Table V

		Tube	1	2	3	4	5	6	7	8	9	10	Controls 11	12
		Dilution	1/25	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25	Organism
Secretion	Test Org.	Time of reading												
Tears	1	1 hr.	—	—	—	—	—	—	—	—	—	—	—	
		18 hrs.	—	—	—	—	—	—	—	—	—	—	—	
	13	1 hr.	—	—	—	—	—	—	—	—	—	—	—	
		17 hrs.	—	—	—	—	—	—	—	—	—	—	—	
Saliva	1	1 hr.	+++	++++	++++	++++	+(+)	(+)	—	—	—	—	—	
		15 hrs.	++(+)	++++	++++	++++	++++	++++	++++	+++	—	—	—	
	13	1 hr.	+++(+)	+++(+)	++++	++++	++++	+++	++(+)	++(+)	++(+)	—	—	
		15 hrs.	+++(+)	++++	++++	++++	++++	++++	+++(+)	+++	+++	—	—	
Colon Washings	1	1 hr.	—	—	—	—	—	—	—	—	—	—	—	
		18 hrs.	—	—	—	—	—	—	—	—	—	—	—	
	13	1 hr.	—	—	—	—	—	—	—	—	—	—	—	
		18 hrs.	—	—	—	—	—	—	—	—	—	—	—	

++++ = Complete lysis. +++(+) = Nearly complete etc.

rat and guinea-pig also are devoid of action on *M. lysodeikticus*. In three experiments cat saliva showed complete lysis in a dilution of 1 in 1600, 1 in 12,800 and 1 in 1600 respectively. In one experiment, however, the saliva showed no appreciable lytic action on the test organisms.

Colon mucus was used in different concentrations. At first colon washings alone were employed, and gave uniformly negative results in six experiments. The concentration of mucus in these washings necessarily varied, and as a control upon the mucus content, estimations of the precipitable protein were done upon each sample; 5 c.c. of the clear washings were taken, and excess of absolute alcohol added; a faint opalescence developed at once, and the following day the flocculent precipitate which had settled out was centrifugalized, and

the supernatant fluid decanted. The precipitate was washed into graduated centrifuge tubes and subjected to a further period of centrifugalization. The packed precipitate was then read off on the scale, and the amount per c.c. calculated. The average amount of precipitated protein was 1 in 500. Similar estimations were done upon saliva diluted 1 in 10, and the average reading was about 1 in 150.

As the mucus in the colon washings may have been too dilute to exert any antibacterial effects, it was decided to investigate the possible lysozyme content of mucus of the consistency obtained when the mucous membrane was stimulated with dilute mustard oil, as previously described; and of mucus obtained by the injection of pilocarpine. About 0·75 c.c. of thick mucus was put into each of a series of small tubes, and then 1·0 c.c. of a suspension (2000 millions per c.c.) of one of several organisms was layered on to the mucus. In some cases the mucus floated to the top. The tubes were incubated at 37° C., and observed for evidence of lysis at ½ hour, 1 hour and 24 hours. No detectable lysis occurred with any of the organisms tested, namely bacteria No. 1, No. 2, No. 3, No. 11, No. 13, *S. fæcalis*, or *B. coli* (Escherich).

It is therefore concluded that mucus secreted by the colon of the cat in response to chemical (dilute oil) and mechanical (manipulation during the securing of washings) and pharmacological stimuli has no lytic properties with reference to the organisms tested.

Three experiments were done on stomach washings, and the results showed a very low lysozome content. Two of the three experiments were negative, and the third showed lysis only as far as a dilution of 1 in 2, although there was slight evidence of lysis against No. 13 in one of the tests which has just been called negative. As in the case of stomach washings from other animals, the possibility of contamination with saliva must be borne in mind. For obtaining the washings the œsophagus was tied just above the junction with the stomach, and a cannula inserted into the pyloric end of the stomach. The stomach was then washed out repeatedly in an endeavour to free it from saliva. Then the specimen for the experiment was secured. These gastric specimens, which were sometimes quite acid, were neutralized before use.

Four specimens of dried* colonic mucosa were tested. In no case was there complete lysis, though in three there was evidence of some lytic power, namely almost complete lysis at 1 in 32 in one experiment, three-quarters lysis at 1 in 4 in another experiment, half lysis at 1 in 8 in a third.

The specimen which showed almost complete at 1 in 32 with organism No. 1, showed also 50% lysis at 1 in 16 with No. 13.

Two experiments were done on dry stomach mucosa extract, and in one case there was complete lysis up to 1 in 4, and three-quarter lysis up to 1 in 4 in the other case.

The experiments on cat's saliva (with one exception) showed the presence

* Preparation of acetone dried tissue extracts: The organ was first perfused with saline to free it from blood. Then it was removed from the carcase, and the mucosa was thoroughly cleaned with saline and wool swabs. Next it was scraped off and put into acetone, which according to Fleming (1922) precipitates the lysozyme along with protein. After 24 hours (or when dehydration was complete) the material was placed in an incubator at 37° C. to remove the acetone, after which it was ground in a mortar. It was then extracted overnight with 20 c.c. saline per gm. of powder.

of a quite considerable quantity of lytic substance.* As cat's saliva is stated not to contain ptyalin, this enzyme cannot be responsible for the phenomena observed. Cat's tears, however, gave no evidence of the presence of lysozyme, whereas human tears (H.W.F.) tested under similar experimental conditions showed lytic powers in very high dilution (1 in 512,000, though complete lysis was not observed beyond 1 in 16,000).

Extracts of the dried mucosa of colon and stomach showed the presence of a small amount of lytic substance—very small as compared with the corresponding organs of the rabbit and guinea-pig and dog.

Dog secretions and tissues.—Experiments were carried out on lines similar to those described for the cat. The material examined were stomach washings and extracts, colon washings and extracts, saliva, nasal secretion and tears. The *colon washings* gave results which suggested a trace only in most cases (in one dog only did we secure as much as three-quarter lysis in a dilution of 1 in 4). *Stomach washings* in two dogs gave complete lysis in dilutions of 1 in 32 and 1 in 4 respectively. *Colon extracts* in three dogs lysed completely in dilutions respectively of 1 in 128, 1 in 281 and nearly completely in 1 in 32 (this last specimen was disturbingly opalescent). *Stomach extracts* gave complete solution at 1 in 8 in two cases. *Saliva* was powerfully lytic, and dissolved the organisms in a dilution of 1 in 100, 1 in 640, 1 in 1280, 1 in 5120 respectively in four experiments. *Nasal secretion* proved effective at 1 in 640 and 1 in 1280 in two experiments. *Tears* lysed in a dilution of 1 in 1280 and 1 in 3200 in two experiments. Comparing the dried extracts of stomach and colon it is clear that the colon mucosa contains much more lysozyme than stomach mucosa, whereas comparison of the respective washings would indicate that the stomach mucosa contains more than the colon. A possible explanation of this contradiction is the difficulty of being sure that no saliva contaminated the stomach washings.

Rabbit secretions and tissues.—The lytic powers of washings obtained during life from the cleaned mucus membrane of stomach, small intestine and distal colon have been compared with those of the same mucosa dried by acetone and extracted with 20 volumes of saline. *Extracts of dried mucosa* from three rabbits gave after 18 hours complete lysis in dilutions of 1 in 8, 1 in 16 and 1 in 32 respectively of the stomach extract. *Small gut extract* lysed completely in 1 in 8, 1 in 256 and 1 in 64. *Colon extract* was much more powerful; complete lysis appeared in 1 hour at 1 in 128, 1 in 32 and 1 in 128, and after 19 hours was complete at 1 in 512, 1 in 256 and 1 in 512, beyond which the dilutions were not carried.

Washings from mucosa obtained as previously mentioned gave the following results: *Stomach washings* against bacterium No. 1 gave about 50% lysis in a dilution of 1 in 2 and against No. 13 no lysis appeared. Another rabbit's *stomach washings* showed complete lysis at 1 in 2, ranging to 50% lysis at 1 in 16 against No. 1 organism and no lysis against No. 13. A third rabbit gave with No. 1 about 50% lysis at 1 in 16, and with No. 13 practically none. *Colon washings* showed complete lysis at 1 in 16, ranging to 50% lysis at 1 in 128 with organism No. 1, and complete lysis at 1 in 32,

* A reference appears in the *Quarterly Cumulative Index Medicus* to a paper by Belikoff and Astrakhanskaya (1929) dealing with the lytic substances in saliva, but we have been unable to consult this.

ranging to 50% lysis at 1 in 128 with No. 13. Another rabbit showed complete lysis at 1 in 2 ranging to 50% lysis at 1 in 64, with slightly less lysis at 1 in 512 with organism No. 1. A third rabbit showed complete lysis at 1 in 16, ranging to 50% lysis at 1 in 64 with organism No. 1.

It is therefore clear that lysozyme is present in considerable quantity in the mucous membranes, especially in the colon, and appears to be present in considerable concentration in the secretions covering the colonic mucous surface. Washings from the stomach show that it is present in that organ, but in much smaller quantity than in the colon.

Saliva was collected from animals anæsthetized lightly with ether by the intravenous injection of 0·5 c.c. of 1 in 1000 pilocarpine nitrate.

A series of three animals had successive samples taken at intervals of a week. The concentrations showing complete lysis after 19 hours varied in one animal from 1 in 20 to 1 in 160, in another animal from 1 in 40 to 1 in 80, and in the third from 1 in 20 to 1 in 160.

Thus the rabbit's saliva possesses lytic powers, but this in comparison with other species is relatively poor.

Guinea-pig secretions and tissues.—The mucosæ from the alimentary tract were treated in precisely the same way, and the extracts and washings tested as before. *Stomach extracts* from three animals all gave complete lysis at 1 in 256, ranging to nearly complete lysis at 512 against organism No. 1. *Small intestine extracts* gave complete lysis at 1 in 16, 1 in 32 and 1 in 16. *Colon extracts* gave complete lysis at 1 in 32, 1 in 32, 1 in 32. *Stomach washings* gave complete lysis at 1 in 4, 50% at 1 in 16; complete at 1 in 8, 50% at 1 in 32; complete at 1 in 8, 50% at 1 in 32. *Colon washings* gave 50% lysis at 1 in 4; no lysis, 50% at 1 in 4 in three experiments.

It is evident that the results as to dried extracts and also washings are the direct opposite to those obtained in the case of the rabbit, since the stomach of the guinea-pig is more powerful than the colon.

Goat secretions: The opportunity occurred for examining the lytic powers of some secretions from a goat. The *saliva*, which was poured out during anæsthesia with ether, was completely inactive as far as lysis was concerned. *Tears*, on the other hand, showed complete lysis up to a dilution of 1 in 640 and three-quarter lysis up to a dilution of 1 in 5120. Washings prepared from a segment of *colon* showed a trace of lysis only in the greatest concentration. The tissues of this animal were unfortunately not dried so that the above results cannot be compared with those from the dried materials of other species.

Conclusion.—No reason can at present be given for the apparently irregular quantitative distribution of lytic substances in the lining and secretions of the alimentary tract. The results are discussed later. As regards technique, it is thought that the method used to test for lysis gives more reliable results than does the embedding method. These are consistent as to relative quantities of lysozyme present in the various parts of the alimentary canal, though the absolute quantities vary from animal to animal. Thus there is consistently more lysozyme in the colonic mucosa of the rabbit as compared with the stomach mucosa. The method unfortunately is not applicable to the demonstration of inhibition.

(B) MUCUS AS A GROWTH-SUPPORTING MEDIUM.

In the previous section it has been shown that mucus as obtained from the intestine of various animals possesses antibacterial properties in very different degrees; in the case of the cat these properties appear to be almost negligible. The question then arose as to whether mucus from any or all of the animals would provide an adequate medium for the growth of such bacteria as we have been considering. Walthard (1895), working with mucus obtained from the cervical canal of the uterus of pregnant women, concluded that the mucus from this site would not enable bacteria to grow in it. Walthard spread a thin layer of mucus on a glass surface, and sowed this with *Staphylococcus aureus*, *Streptococcus pyogenes* and *B. coli* in visible quantities. A control sowing was made on agar slopes. After incubation for 14 days he read the results by a naked-eye observation of the two surfaces. The method, however, does not seem sufficiently delicate for the detection of the smaller though quite appreciable degrees of growth which may have occurred. However, as the following experiments show, this negative finding does not apply to the mucus of the cat's colon. The technique whereby an enumeration of colonies is taken as an index of the numbers of such bacteria sown on a plate is admittedly liable to serious error, but when two successive counts on any given suspension of bacteria in a fluid medium differ as widely as x and $10x$, or x and $20x$, it is not too much to accept the evidence as showing a true and unmistakable increase in the bacterial population of the medium. The reliability of the method depends largely upon securing thorough separation of the bacteria in the suspension which is to be used, and upon thorough mixing of the ingredients and the diluent prior to sowing the plates. In a fluid containing mucus this thorough mixing is naturally somewhat difficult to ensure, but by using the clearer less viscid portions of any specimen of mucus this difficulty is reduced to a minimum. Briefly the procedure was as follows: Mucus was secured from a cat's colon by stimulating it with dilute mustard oil. As clean a sample as possible, *i.e.* free from oil and gross *débris*, was heated in a water-bath at 60°C. for 1 hour on three successive days. This temperature was chosen as most likely to leave proteins unaltered while ensuring sterility of the mucus. The "sterilized" mucus was then divided into measured portions, and to each was added a definite quantity of an emulsion of one of several living organisms. The mucus and the emulsion were mixed as thoroughly as possible, and a small sample was then withdrawn, diluted and plated out. Suitable controls were also plated out to test the sterility of the mucus. The inoculated mucus was incubated at 37°C. overnight, and next day a second sample was withdrawn, diluted and plated out as before. The numbers of colonies obtained from the first and second samples were compared. Using two different specimens of mucus tests were set up with *B. coli*, *B. prodigiosus* (twice), *M. lysodeikticus* (twice) and *S. lutea*. In all cases the second sample from the inoculated mucus showed an increase in the number of organisms as judged by colony counts; the increase ranged from 5- to 25-fold in three experiments where convenient dilutions permitted a count of colonies to be made; in three others the increase was unmistakable, but the colonies were

too numerous to count. The mucus controls showed only a few contaminating colonies or were sterile.

Two experiments on somewhat similar lines were done, using, however, fresh unheated mucus from a cat's colon. No special organism was inoculated into the mucus, but the original bacteria present in the mucus were utilized as indicators. In one experiment fresh mucus (secured from a cat's colon after massive doses of pilocarpine) was diluted 1 in 5 and 1 in 50 with sterile normal saline. The bacteria in the undiluted and diluted specimens were estimated by colony counts, firstly before incubation, and secondly after 24 hours' incubation at 37°C. The second count showed an increase of about 500-fold in the bacterial flora. In the other experiment mucus similar to that used above was diluted 1 in 10, 1 in 100 and 1 in 1000, and treated as in the previous experiment. The increase in the bacterial population, after incubation, was about 1000 fold.

The conclusion drawn from this series of experiments is that mucus as secreted by the cat's colon in response to stimulation by dilute mustard oil or pilocarpine is capable of supporting the active growth of certain bacteria. Admittedly the mucus contains *débris* from the epithelial lining of the colon, and occasionally small particles of the normal contents of the lumen, for we were unable to secure a sufficiently large specimen of mucus free from these inclusions to carry out tests on "pure" mucus. Indeed, it seems unlikely that any considerable quantity of mucus could be collected free from epithelial *débris*. However, our experiments warrant the tentative conclusion that certain bacteria are capable of growing in the mucus secreted by the cat's colon.

(c) *IN VIVO* EXPERIMENTS.

Several investigators have examined the possible antibacterial properties of the normal living mucous membrane of the intestine (*e.g.* Arnold, 1929, Eiskamp and Park, 1921), and the general conclusion seems to favour the existence of some unspecified antibacterial mechanism resident in the intact mucosa.

Our examination of mucus has not yielded any evidence of significant antibacterial substances in that of the cat at least. A few experiments were therefore carried out with a view to controlling the susceptibility of our test organisms to this ill-defined mechanism of bacterial control.

A Thiry fistula of the distal portion of the colon was prepared in a cat. When the cat had completely recovered, *aërobic* cultures were made from the colonic fistula on several successive occasions at a few days' interval. Then an emulsion of an organism not present on any of the preliminary normal control plates, and not normally present in the intestine, was injected into the fistula. Thereafter at intervals cultures were made in order to trace the rate of disappearance of the injected organisms. Using *M. lysodeikticus*, a few colonies were recovered 24 hours after injection.

A dog with an ordinary Thiry fistula of the colon inserted about two years previously was treated similarly to the cat. *B. prodigiosus* was injected in this instance, and there was a rapid progressive diminution in the numbers of this organism recovered, together with an apparent progressive relative increase in the numbers of *B. coli*. There were still some *B. prodigiosus* present after 24 hours.

It is of some interest to note that *B. coli* has persisted in the fistula for more than two years, although this has been quite cut off from the alimentary tract during this time—a fact which supports the previous conclusion that colonic mucus may act as a medium of growth. In general, the results confirm those of other investigators (Arnold, 1929; Eiskamp and Park, 1921). There is a great reduction in the numbers of the injected organisms after a lapse of several hours.

(D) OTHER POSSIBLE PROTECTIVE PROPERTIES.

The search for definite antibacterial qualities in mucus has given us discordant results, *e. g.* the mucus in the colon of the rabbit is rich in lysozyme, whereas that of the cat seems to be devoid of detectable quantities. It appears, therefore, that any protective action of mucus common to all species, against the entry of bacteria from the lumen of the gut into the tissues must be sought rather in its mechanical properties than in its ability to destroy bacteria. The mechanical barrier offered by the mucous secretion of the colon of a cat was well seen by the following means: with fine scissors a very small portion of the surface of the mucosa was snipped off and placed on a slide in a drop of saline. A coverslip was then brought down on it to crush the fragment, and the edges were sealed with paraffin or vaseline. A large number of vibrios were present in all specimens examined. These were seen to dart about rapidly, but their movements were arrested by contact with the viscous mucus in the neighbourhood of the cells. Clearly, then, mucus opposes a mechanical barrier to the penetration of bacteria and their further passage towards the cells. When, however, the mucus-producing cells of the colon are destroyed by X-rays or radium (Mottram and Kingsbury, 1924) or vitamin A deficiency (Cramer and Kingsbury, 1924), bacteria in considerable numbers are able to invade the crypts and enter the circulation. During normal life there is a constant slight secretion of mucus, even when no food substances are present in the lumen of the gut, *e. g.* a Thiry fistula pouch of the colon of a dog continues to secrete mucus even in a completely resting state. This slight secretion of mucus is immediately increased, possibly enormously so, by mechanical, thermal or chemical irritation. Hence any increase of bacteria producing irritating substances would cause the outflow of more secretion, thus carrying the source of irritation away from the cells themselves. Thus when no bactericidal factor operates, this mechanical action of mucus would seem to be the principal means of protection against the invasion of bacteria. Not only is the normal layer of mucus too viscous to permit penetration by bacteria, but also this normal layer is increased in thickness by irritation.

DISCUSSION AND SUMMARY.

We owe the original description of lysozyme and its properties to Fleming and his co-workers (1922). This lytic substance is of enzymic nature (Fleming and Allison, 1922; Wolff, 1927a and 1927b; Hallauer, 1929), and was found to be widely distributed in various species of animals, and even in vegetables. Of the mammalian secretions examined by these workers, human tears were found to be the most powerfully lytic to *M. lysodeikticus*, whereas human

sputum and nasal mucus were both very effective and saliva considerably less so. They drew from their observations the conclusion that this enzyme was of great importance in keeping in check possible microbic invaders, and related the substance to some process of natural immunity.

Our observations have been chiefly concerned with an investigation of the lysozyme content of the mucous secretion of the alimentary tract—a very common portal of entry for micro-organisms. The cat was the animal upon which the bulk of the experiments were done, and by reference to our figures and tables it will be seen that cat's tears are entirely devoid of lytic properties when tested against sensitive air bacteria. Saliva, on the contrary, usually contains a considerable amount of lysozyme, but we have been unable to detect it in mucus procured from the stomach or colon, though a small amount as compared with other animals can be demonstrated in the saline extracts of dried mucosæ.

On the other hand, marked lysis was effected with dog's tears, saliva and nasal mucus, and there were detectable amounts in the mucus membranes and washings, much more being present in the colon mucosa than in stomach mucosa.

Three herbivorous animals were also examined—rabbit, guinea-pig and goat. The rabbit manifested very marked lytic powers in both washings and extracts of mucosa; the colon was again much more active than the stomach, and small intestine fell midway between the two. Rabbit's saliva was active to a much less degree than colon extract. The guinea-pig, *per contra*, reversed this relationship, stomach being more potent than intestine. The goat (one only was available) showed a complete absence of lysozyme from its saliva in a dilution of 1 in 10. Colon washings showed a mere trace of lysis; tears, on the other hand, were quite active.

It is thus clear that no generalization can be made as to the site and concentration of lysozyme when judged by the experiments on these five species. Though undoubtedly present in the tissues of all animals examined, those of the cat are especially poor. Of all the secretions investigated in this series of animals, the saliva appears to contain the lysozyme most constantly. However, the goat is not supplied with this possible protecting mechanism, yet its conditions of life and anatomical arrangements of the alimentary canal are not obviously different from those of the guinea-pig and rabbit.

Our data concerning the distribution of lysozyme along the alimentary tract do not enable us to envisage any definite mechanism, since in some cases the mucosa of the colon, and in others of the stomach, is most powerfully lytic; while in others again there seems to be very little lysozyme at all. We are therefore of the opinion that though the lysozyme content of these various tissues and secretions may be of some importance in the resistance of the animals to infection, it is necessary to obtain many more data as to its distribution on an anatomical basis, and also data as to the chemical nature of the substrate on which the enzyme acts, before being certain that it serves a purely antibacterial function. It is conceivable that it is accidental from the immunological point of view that the lysozyme destroys a number of susceptible bacteria. Thus not even all air organisms are destroyed or even inhibited, and according to Fleming, very few pathogens are affected.

APPENDIX: INHIBITION OF ONE BACTERIUM BY ANOTHER.

This is a very well-known phenomenon, and has been described mainly for pathogenic organisms (Lode, 1903). Papacostas and Gate (1928) review this subject at length. It was therefore not surprising to find that a phenomenon of this nature had developed on a plate prepared with intestinal mucus. The particular plate which forced this fact upon our notice showed a small area of complete absence of growth of the test organism over a large single colony lying at the extreme edge of the embedded mucus. To extend this observation, we subcultured the colony, and set up experiments aiming specifically at an investigation of its inhibitory properties. For this inquiry the following

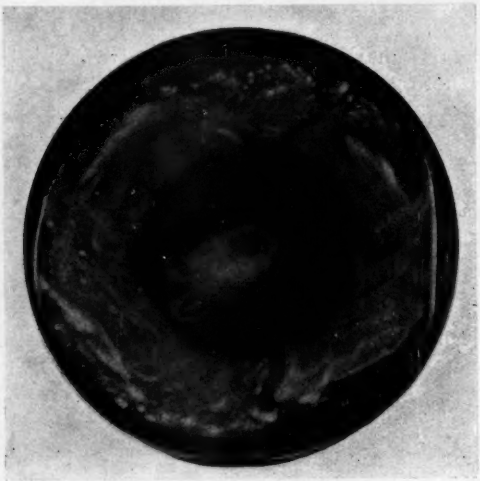

FIG. 2.—Inhibition of bacterium No. 1 by *B. coli* (Escherich). The central colony of *B. coli* is on the under-surface of the agar. Bacterium No. 1 is on the upper surface. (Photo by H. P. Hudson.)

technique was developed: On an ordinary agar plate a small circular area about $\frac{5}{8}$ in. diameter was sown centrally. After 24 hours' incubation the agar was cut about $\frac{1}{4}$ in. from the edge of the Petri dish and carefully inverted into another Petri dish. The test organism was then sown broadcast on the surface thus exposed, and the plate incubated. After 24 hours readings were made. In the case of this organism it was found that complete inhibition over an area intimately related to the area occupied by the inhibitory organism had occurred with organisms Nos. 1, 11 and 13, and well-marked, but incomplete, inhibition with Nos. 2 and 3 (see Fig. 2). As a further extension of these observations, four different organisms not identified and named here, *A*, *B*, *C* and *D*, were isolated from cat's fæces and tested in similar manner for inhibitory influence upon the usual test organisms. A number of fæcal

bacteria were obtained from the National Collection of Type Cultures, and results of observations on these are included in Table VI. It was thus clear that bacteria existing in the colon were able to inhibit the growth of the

Table VI

Test Organism	B. Neapolitanus	B Communis	B – Ac Lact	B.c.c. (Escherich)	B.c.c.	Strep. faec	B. Proteus	B Cloacae	B Lact Aerog	Gut A	Gut B	Gut C	Gut D	Gut E	B "Adden" *
1	—	—	—	+++	(–)	—	(+)	+	++(+)	—	—	—	—	+++	+++
	—	—	—	++(+)	—	(+)	(+)	—	(–)	—	c	+	+(+)	+++	
	—			++(+)	—	—	—	c	++(+) +++	—	(+)	(+)	— —		
B. Coli														=	
2	—	—	++	—	(–)	—	++	—	—	++(+)	c	—	(+)	++	(+)
	(+)	(+)	—	(+)	—	(+)	(+)	+	—	++	+	—	+	++(+)	
	—		+	+	—	—	— (+)	—	—	(+) (+)	+	—	—		
3	—	—	—	—	(–)	++(+)	(+)	++	—	++(+)	+	—	—	++	—
	—	—	—	—	—	—	—	(+)	—	lost	—	(+)	(+)	++(+)	
	—	—	—	—	—	—	(+)	(+)	—	+	+(+)	—	(+)		
	—			—		—	+	lost	—	c	(–)				
						—		(+)							
11	++(+)	+	—	—	(–)	+++	—	+++	—	+++	—	+++	++(+)	+++	+++
	—	+	—	—	++	++(+)	lost	—	—	lost	++	++(+)	c	++	
	(+)	—	—	+(+)	—	++	+	++(+)	(–)	+++	—	++(+)	++(+)		
	—			c	—	++(+)	—	—	—		—				
	+			—				+							
13	—	—	—	++	(–)	—	—	+++	++(+)	—	+(+)	+++	+++	+++	+++
	—	—	—	++(+)	++	—	—	++	++(+)	—	++	+++	+++	+++	
	—	—	—	++(+)	—	—	—	++(+)	++(+)	—	+(+)	++	+++		
	—			++(+)	—	++(+)		++(+)	++(+)						
B Prodig.				—							—				

* A bacillus isolated from sputum (human) +++ = Complete inhibition

++(+)=Almost complete inhibition ++ = Some inhibition + =Noticeable thinning of growth

(+) = Appearance of roughness (–) = Just appreciable roughness c = Contaminated

test organisms in a way indistinguishable from that of lysozyme when examined by the method of tissue embedding and "well" plates. The mechanisms of these inhibitions have not been investigated, but it seems possible from similar results reported in the literature that a variety of causes

was operating. It is of interest, however, to note that *M. lysodeikticus* is more consistently and completely inhibited by a larger range of organisms than is any other of the bacteria tested, namely Nos. 1, 2, 3 and 11.

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FURTHER INVESTIGATIONS ON THE INDUCTION OF TUMOURS WITH CARBON DIOXIDE SNOW.

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IN a previous paper (1929) it has been shown that repeated mild freezing of the skin of mice with carbon dioxide snow may lead to the development of both simple and malignant tumours in a small percentage of cases. Freezing with carbon dioxide snow may therefore be considered as a mild carcinogenic agent. In a further experiment, it was shown that when an area of skin was subjected both to freezing and to tarring, the rate of tumour-formation was, on the whole, no greater than when tarring alone was used, but the distribution of the tumours was somewhat characteristic. Several of the warts arose outside the frozen area, owing to the fact that the tar invariably spread beyond the frozen area to which it was originally applied; the majority of the warts developed at the immediate margin of that area, but no warts appeared in the area itself where the freezing and the tarring overlapped. In other words, when two carcinogenic agents

were applied to the same area of skin, the result was not an augmentation of carcinogenesis, but actually an inhibition. The suggestion was put forward tentatively that the irritation may have been too severe for tumours to develop. It would appear that an excessive amount of irritation, whether produced by one agent or by two, is as unfavourable as an insufficient amount, from which it follows that there is an optimum intensity of irritation for the induction of tumours.

If the failure to induce tumours by applying carbon dioxide snow and tar concurrently was due to the fact that the irritation was too severe, it should be possible, by applying the two agents separately during two consecutive periods, not only to produce tumours successfully but to get a summation of effect. This hypothesis was tested in the following experiments:

Employing the technique of the experiments described in the previous communication, 50 mice were tarred once a week for 9 weeks; the tarring was then discontinued, and carbon dioxide snow was applied two or three times a week to the same area of skin for a further 12 weeks. The result was the development of tumours in 9 animals. In 2 animals out of the 9 the warts appeared outside the frozen area; in 5 they developed at the margin of the frozen area, while in the remaining 2 mice the warts appeared in the frozen area itself; 6 of these warts regressed; 2 grew slowly and remained simple, and 1 grew rapidly and became malignant. In a control series of 50 mice, in which the tarring was not followed by any subsequent treatment, only one mouse developed a wart, which dropped off after a short time (Table I).

TABLE I.

	Tar for 9 weeks, then CO_2 snow.	Tar for 9 weeks.
Number of survivors at the end of tarring	42	42
Total number of animals bearing tumours	9	1
Times of first appearance of tumours*	1, 1½, 1½, 3½, 4, 5½, 7½, 9½ and 9½ weeks respectively	½ week
Number of survivors 9½ weeks after cessation of tarring	27	33
Number of mice in which warts became malignant	1	0

* Counted from the time the tarring was discontinued.

Though these results seemed fairly conclusive, it was decided to repeat the experiment, but with certain slight modifications.

In the second experiment 100 mice were tarred once a week for 12 weeks, by which time one mouse was found to have developed a wart. This animal was discarded, and of the remaining 48 survivors,* 24 were set aside to act as controls and given no further treatment, while the remaining 24 received weekly applications of carbon dioxide snow for 6 weeks, after which no further treatment was given. In the control series only 2 mice developed warts. These

* The mortality was fairly high in the early part of this experiment.

warts grew progressively, but had not attained malignancy by the 30th week, when all the animals were killed for histological examination. Among those animals which received carbon dioxide snow treatment subsequent to the tarring, 13 mice developed tumours (Table II). In 4 of these the warts developed outside the frozen area; in 7 cases they appeared at the margin, and in the remaining 2 cases they developed within the frozen area; 4 of the animals with tumours died very early in the experiment; in 2 cases the warts tended to regress; in 3 cases they remained stationary, whereas in the remaining 4 they grew progressively; 2 of these became malignant.

TABLE II.

	Tar for 12 weeks, then CO_2 snow.	Tar for 12 weeks.
Number of survivors at the end of tarring	24	24
Total number of animals bearing tumours	13	2
Times of first appearance of tumours*	1, 1, 1, 1, 1, 2, 2, 2, 3, 5, 5. 5 and 7 weeks respectively	2 and 3 weeks respectively.
Number of survivors 7 weeks after cessation of tarring	13	15
Number of mice in which warts became malignant	2	0

* Counted from the time the tarring was discontinued.

In short, these experiments show that the induction of tumours following a limited period of tarring is greatly increased if carbon dioxide snow is applied to the same area of skin after the applications of tar have been discontinued.

DISCUSSION AND CONCLUSIONS.

When two carcinogenic agents—tar and carbon dioxide snow—are applied concurrently to the same area of skin the induction of tumours is inhibited, but when each is applied alone for a certain period, a summation of effect is obtained. This confirms the previous supposition that when the intensity of irritation is too great, the power to induce tumours is impaired. It also shows that the changes in the tissues previous to the actual appearance of a wart are not specific for the different carcinogenic agents, but that such agents can be interchanged during the pre-wart period, so that the effect of one is added to that of the other, with the subsequent development of tumours.

It is generally held that a carcinogenic agent is ineffective if the intensity of irritation is insufficient. The present experiments suggest that it may also be ineffective if the intensity of irritation is too great.

A carcinogenic agent consisting of a mixture of different chemical substances (*e. g.* tar) can be rendered more potent by fractional distillation or by chemical extraction (Twort and Fulton, 1930, and others). When two fractions are obtained by any such method, one being more potent than the other, it is generally assumed that the more potent one contains a greater amount of "specific carcinogenic principle" than the other. But since there appears to be an optimum intensity of irritation for effective carcinogenesis, it may be

that the weaker fraction is less potent because it contains an excess of carcinogenic principle rather than a deficiency. Theoretically, therefore, a "concentrated" fraction is one which contains an optimum concentration of carcinogenic irritants and not necessarily a maximum concentration.

The present investigations have also an important bearing on the use of carbon dioxide snow for therapeutic purposes in man. Although carbon dioxide snow by itself is a weak carcinogenic agent, producing tumours in few cases and only after many months of treatment, a single application can lead to the development of tumours if made on an area of skin previously treated with tar. In other words, a single application of carbon dioxide snow to an area of skin which is in a pre-cancerous condition may precipitate the development of a tumour. In the previous paper (1929) it was suggested that carbon dioxide snow is not likely to induce tumours in man unless applied repeatedly for more than twelve years. This may be true, but only in the case of healthy skin. It is not unreasonable to conclude from the present experiments that the use of this agent for the treatment of pre-cancerous lesions of the skin may not be entirely devoid of risk.

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ROUS SARCOMA No. 1: INFLUENCE OF MODE OF EXTRACTION ON THE POTENCY OF FILTRATES.

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IN a previous paper (Gye and Andrewes, 1926) it was shown that the property of filterability of the Rous sarcoma No. 1 is variable, and that the firm, yellow, relatively benign forms of the growth are usually non-filterable. It is proposed in this paper to report further observations upon the filterability of the tumour. Details of individual experiments will not be given since simple crucial tests are difficult to devise and carry out. We wish rather to give conclusions derived from a long and close acquaintance with the Rous sarcoma; we wish to give the general results of a review of a large and varied series of experiments originally made with very divergent objects, rather than to describe one or two specially made experiments which in themselves must be quite inconclusive. Illustrative experiments could be given but they would have to be selected experiments, *i. e.* experiments selected because, on the basis

of our general experience, we are satisfied that the correlations suggested by the results are not misleading. In respect of the particular questions we propose to discuss, the confident interpretation of any single experiment or even of any short series of experiments is wellnigh impossible apart from the help of a much wider experience, because of the magnitude of uncontrollable accidental influences which may affect individual results without being detected.

Different specimens of the Rous sarcoma No. 1 vary considerably in both macroscopic and microscopic appearance, and it has been observed that there exists a rough correspondence between these various appearances and the various degrees of filterability presented by the tumour. The so-called classical form of the Rous sarcoma No. 1 is a rapidly growing, hæmorrhagic new-growth which contains one or more cavities in which blood, tissue *débris* and slimy fluid accumulate, and into which project fungating masses of malignant tissue. It is often difficult to separate the tumour tissue from muscle and other normal tissues owing to the failure of the tumour to grow as a discrete mass. When metastases occur they are most commonly found in the lungs, less frequently in the liver and heart muscle. Sometimes the secondary deposits in the liver consist of small rounded hæmorrhagic masses, and these are mostly scattered over the surface of the organ. The classical type of the Rous tumour causes the death of a half-grown Plymouth rock fowl in from 20 to 30 days.

The firm, yellow, slow-growing type of tumour which has previously been described (Gye and Andrewes, 1926) occurs sporadically from time to time, its occurrence in normal chickens depending upon a pre-existing resistance in the bird. Whether this resistance is a resistance to implanted cells or to the infective agent associated with the cells, or to both together, we have not determined. Rous (1913) brought forward evidence that both forms of resistance to this transplantable, filterable fowl tumour may exist independently.

A third form of the Rous Sarcoma No. 1, also occurring irregularly, is more malignant than the "classical" common type. In this form of growth the implanted cells grow with such energy that the muscles into which implantation has been made are rapidly replaced by an almost solid mass of new-growth. Microscopic examination of such a tumour shows that the delicate, sarcomatous spindle-cells are widely separated from one another by large areas of necrosed muscle. This highly malignant tumour is sometimes red in colour, occasionally buff; it can be removed in slices, and is not accompanied by the blood-stained, sticky fluid so characteristic of the average form of growth. These very malignant tumours kill a young fowl in from 12 to 20 days.

This attempt to separate the tumour into three types is artificial; it must be understood that the types merge into one another. Even in a single specimen a transition of types may be seen. A firm yellow tumour, for example, may grow slowly for many weeks and then suddenly begin to grow rapidly, usually from one pole. In such a case it is easy to distinguish post-mortem the new, rapidly growing, usually hæmorrhagic tissue from the old, firm, yellow tumour. But although the classification is only a rough one, it is a matter of practical importance to recognize these different types of tumours formed by different modes of growth, since the more rapidly growing and the more malignant a tumour is, the more likely is it to yield a highly potent

filtrate. If these indications are neglected, studies directed towards an understanding of the nature of the infective material of active filtrates are hampered to an unnecessary degree by the frequency with which non-potent filtrates are obtained.

In an extended series of experiments made in the course of various investigations the method of extraction has been standardized, and it has been shown that the variable-filtrability is a real phenomenon connected with the constitution of the tumour itself, and is not to be confused with those wide but chance variations which are due simply to unavoidable differences in the filters used; but while it is perfectly certain that the tumours can be arranged in a continuous series according to the grade of potency possessed by the filtrates which they yield, yet to say that a tumour is absolutely non-filterable is justified only with the qualification "by the methods employed," as will be shown.

In the experiments filter-candles are used to ensure that the filtrates shall be really cell-free; but candles themselves very greatly reduce the potency of extracts passing through them. To such an extent is this so that it is relatively frequent to find that a cell-free candle filtrate of somewhat low potency is rendered entirely inactive if it be passed through a second candle. When a candle has been used, therefore, it is impossible to say with confidence that because the filtrate is inactive it has been proved that no agent separable from the tumour-cells can have been present in the unfiltered extract; small amounts of agent can be completely absorbed by the candle. That candles are responsible for the shifting of the zero point of infectivity is proved by experiments in which extracts are spun at high speed, but not filtered. Such spun extracts may be entirely inactive when injected into chickens, even in large doses. We admit the difficulty in being *absolutely* certain that in any given spun extract *no* cells can be present, but simple spinning is permissible here since the failure to remove every living cell could only lead to a non-filterable tumour being wrongly classed amongst the filterable ones; the negative experiments stand unaffected.

It follows from what has been said already that in order to reduce the drop in potency to a minimum repeated filtration must be avoided. Each filter levies its toll. Filters made of paper-pulp and sand such as that described by Gye and Andrews (1926) do not reduce the infectivity as much as candles do, but unless they are skilfully made and used, cells and cell *débris* may appear in the filtrate. It is at present our practice to clear an extract as far as possible by spinning, and then, as best suits the needs of the experiment, pass the spun extract either through a paper-pulp and sand filter or else through a Berkefeld candle.

But certain observations suggested that besides being considerably affected by the filters used, the potency of a filtrate may be greatly influenced also by the method of extraction used. For instance, a review of a long series of experiments indicated that both distilled water and phosphate solutions are less efficient as extractives than is physiological saline. This side of the technique of the preparation of cell-free extracts therefore merits attention.

It is easy to obtain very active cell-free extracts of the rapidly growing form of the Rous tumour. Tumour-tissue is broken up with sand and is then

extracted with normal saline. A proportion of tissue to extracting fluid of 4 to 5 gm. per 100 c.c. has been found to be satisfactory. The method originally employed by Rous consists of spinning the turbid extract in order to deposit the larger particles of tissue *débris*, and then filtering through a Berkefeld candle. We have found that this method gives active filtrates when large candles (120 mm. × 25 mm.) are used. With small candles (50 mm. × 15 mm.) it is difficult to obtain more than 30 to 40 c.c. of filtrate, and in our experience this filtrate is usually not highly infective.

The difficulty of preparing active cell-free extracts is greater with the slow-growing tumours, though it is true, as was pointed out by Rous, that rapidly-growing tumours sometimes fail to yield potent filtrates; such results are inexplicable. Filtrates made from slow-growing, firm tumours are usually inactive or only barely active, rarely very active. The problem which arises from these general observations is whether the failure to obtain active filtrates from such tumours is due to an "attenuation" of the virus, or whether the virus is present only in small numbers, or whether again the technical methods of extraction which are suited to highly malignant tumours are less suitable and adequate to the firm, almost benign new growths. The hypothesis that the virus is "attenuated" will be shown below to be wrong; we have no means of estimating the number or quantity of virus, and the hypothesis of its diminution cannot therefore be investigated, but this and the possibility that the infective material is more tightly bound up with the cells in slow-growing tumours are both hypotheses which are tenable on the basis of the following observations.

During the course of numerous experiments which were carried out with various primary objects, it was observed incidentally that a rough correspondence exists between the amount of protein which may be precipitated from filtrates by means of acetic acid in the cold and the degree of infectivity of the filtrates. The greater the amount of precipitable protein, as a rule, the greater the potency of the filtrate from any given tumour. The precipitate obtained consists partly of mucin and partly of "nucleoprotein." When relatively benign tumours are extracted with a saturated solution of sodium chloride, and this extract is then diluted with distilled water until the salt is reduced to the strength of physiological saline, it is found that the filtered extract contains an abundance of protein—much more than if a like amount of tissue is extracted with saline in the ordinary way—and that usually parallel with this increase in protein content the potency of the filtrates is increased. The potency of ordinary filtrates of highly malignant, rapidly-growing tumours is already so high that any increase due to extraction by the saturated salt method would be extremely difficult to detect, but with tumours of slower growth the effect is obvious; often, indeed, by this saturated salt method, potent filtrates may be obtained from tumours which are otherwise non-filterable.

The natural question that arises in this connection is whether the virus in these extracts from hard, slow-growing tumours is of a modified character. Two experiments are worth description in this connection. In these experiments the tumours which were used to provide the filtrates were of very low malignancy and, in fact, had been specially selected because of the probability that by ordinary methods of extraction they would have failed to yield potent filtrates.

Experiment of September 11th, 1928.—A slow-growing, firm white Rous sarcoma was taken; 2 gms. of the tissue was broken up with sand, and 1·2 c.c. of a saturated solution of sodium chloride added. To the sticky mass which was produced, 50 c.c. of sterile distilled water was now added slowly, the mixture being stirred continuously. After spinning, the fluid was filtered through a Berkefeld N candle. The filtrate was colourless, and when a sample was boiled and acidified a flocculent deposit was produced. This filtrate was injected into 6 chickens in doses of 0·5 c.c.; in each bird a tumour ensued, growing rapidly and killing the host. One of the chickens died in 17 days, three in 19, one in 26 and one in 29 days. In each case the tumour was of the classical malignant type.

Experiment of September 20th, 1928.—The tumour used in this experiment was firm and white, with a recent hæmorrhagic, highly malignant extension from one part. The firm bloodless portion was separated carefully from the more recent growth, and from it a filtrate was made by the same method as was employed in the experiment described above. The colourless filtrate was injected in 0·5 c.c. doses into three half-grown fowls. In each bird a tumour formed and grew rapidly. One bird died in 18 days, one in 20 and one in 21 days. The tumours were in each case typically malignant hæmorrhagic growths.

It will be noted that the tumours induced by the filtrates obtained by this method of extraction were much more malignant than the tumours from which the filtrates were made. From this fact the conclusion is permissible that the tumours which grow slowly, and which, as a rule, yield inactive filtrates when extracted with physiological saline, do not owe their relatively benign properties to an attenuation of the virus. The virus is shown in these experiments to be normally "virulent." This result of the inoculation of filtrates is quite a common one, and it may be contrasted with the observation that when living cells are used as the inoculum the resulting daughter-tumours always tend to resemble the parent-tumours. Grafts of these firm, slow-growing neoplasms usually give rise to similarly slow-growing, firm daughter-tumours, although a tendency to revert to the normal, more malignant type is frequently observed.

The practical advantage of this more efficient means of securing potent filtrates need not be insisted upon. All who have worked with the Rous sarcoma must have lost much time and many birds through the occurrence of non-potent filtrates. By the use of the method of extraction which has been indicated this loss may be reduced considerably. But the fact that many otherwise non-filterable tumours become filterable when extracts are made by more efficient means is also of wider significance. The increased potency of extracts appears to be due to the more complete disruption of the tumour-cell and the increased amount of protein dissolved by strong salt concentrations. Since a large part of the increased amount of protein extracted comes from cell nuclei, it is permissible to hazard the suggestion that the infective material of the tumour is bound up with the nucleus. It is impossible to decide whether the further inference may be made that in non-filterable tumours the active material is too small in amount to give extracts above the threshold value of infectivity, or whether the infective material is too tightly bound with cells to be extractable by any method so far discovered. The further possibility that inhibitory substances exist in quantities variable in relation to quantity of active agent

present in the tumour-cell has been explored, but the results of experiments are so far inconclusive.

The artificiality of the classification of Rous tumours as filterable and non-filterable is clearly indicated since the classification is shown to be dependent upon the efficiency of the technical methods employed. Even when tested by the best methods we can now devise, there still remain, however, examples of the Rous Sarcoma which are non-filterable. Therefore the remark made in the preceding paper is still pertinent—"It is inadmissible to argue that filterability constitutes a real difference between the Rous fowl sarcoma No. 1 and other transplantable tumours."

CONCLUSIONS.

1. There is a rough correspondence between the structure of each example of the Rous sarcoma No. 1 and its degree of filterability.
2. There is a rough correspondence between the potency of an extract and the amount of precipitable protein that it contains; but some specimens yield extracts that are very highly potent, although they contain no protein detectable when tested by boiling and addition of acetic acid.
3. Extraction with strong salt and subsequent dilution to physiological strength gives more highly potent extracts than does direct extraction with physiological saline.
4. By the saturated-salt method some examples of the Rous sarcoma No. 1 which once would have been classed as non-filterable are now found to be filterable.
5. Since it is shown that in practice the classification is dependent upon the efficiency of the technical tests employed, added point is given to the question of whether the strict separation of tumours into filterable and non-filterable groups is not entirely artificial.

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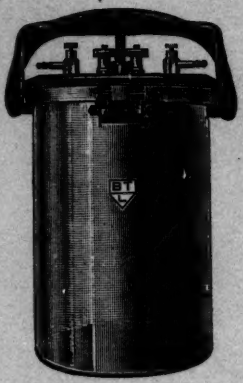

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